

METHOD OF PRODUCING RECOMBINANT DNA MOLECULES**FIELD OF THE INVENTION**

The present invention is directed to an improved method for producing by recombinant methods proteins that occur in nature in two or more subunits; more generally, it is applicable to the amplification and subsequent expression of any chimeric DNA molecule that results from the ligation of two or more non-contiguous pieces of DNA.

BACKGROUND OF THE INVENTION

The expression of fusion proteins is well known in the art and it is disclosed for instance in the following patent publications: EP-6694, EP-20290, US-4898830, US-5452199, EP-213472, EP-196864, EP-461165.

The manufacture of recombinant proteins of interest (e.g., human) in suitable expression systems is one of the main industrial applications of recombinant DNA technology. If, as is often the case, the protein is unstable in the host cell, it may be advantageous to manufacture the protein of interest in the form of a fusion moiety comprising a protective (or stabilising) protein which will be subsequently processed at a specific predetermined site in order to free the desired protein.

Another reason for making fusion proteins is to increase expression levels and/or to facilitate the purification process by the selection of suitable polypeptide sequences and attachment of one or more of such sequences to the amino- or carboxy-terminal ends of the polypeptide or protein of interest. A further reason for expressing a fusion protein might be that of having the characterizing features of two or more different proteins or subunits in a single chain, thus providing a higher activity/dose ratio of the protein itself, and/or avoiding the extra steps to obtain ligation of two subunits. One of the classical problems associated with the expression of recombinant proteins is that of obtaining a valuable and reliable source of the nucleic acid to be expressed.

One way of addressing this problem is to use an mRNA coding for the protein to be expressed. mRNA is however not always easily found under natural conditions. For example, in the case of beta-chain human follicle stimulating hormone (FSH), the corresponding mRNA can be found only in human pituitary cells and only in minute quantities. To be useable, this mRNA must be taken from the human pituitary cells immediately after death.

An alternative is to obtain the coding sequences of interest from genomic DNA. This is however a cumbersome and time consuming process as often, large amounts of unwanted DNA material are present in the initial sample which increases the probability of mutations and other errors. The need therefore still exists for an improved process for generating nucleic acid sequences to be used for expressing polypeptides, and in particular for generating nucleic acid sequences to be used for expression of heterologous recombinant fusion polypeptides.

SUMMARY OF THE INVENTION

The present inventors have now found a method that permits the expression of proteins and fusion proteins starting from genomic DNA without at least one of the foregoing disadvantages associated with known methods. This new methodology offers a novel approach to the production of recombinant proteins: the present methodology makes it possible to ligate and amplify encoding pieces of DNA at the desired positions without the use of restriction enzymes. This means that it is possible to use only the exact encoding regions of a desired DNA (i.e. without introns) thus avoiding splicing and/or reducing the formation of non desired amplicons.

An example of the advantages of this new methodology is represented by the expression of the human beta FSH. The gene expressing human beta FSH is over 1500 bases long whereas the corresponding coding region is only 390 bases long. According to the present methodology it is possible to construct an expression construct containing the correct 390 bases DNA sequence without having to use the 1500 bases of the entire gene. It is thus possible to eliminate

1110 extra bases of unwanted and possibly problem causing DNA without using restriction enzymes and without going through the extra steps of isolating mRNA, and generating cDNA. The present invention is thus expedient, inexpensive and less error prone.

With the present methodology it is also possible and relatively simple to design chimeric molecules of DNA expressing the characteristics of two different proteins or protein subunits in a single chain or even expressing only portions of a particular protein responsible for its activity. For example it is possible to express a new protein having FSH activity as well as LH activity. In other words, it is possible to create new proteins with additional, increased or otherwise modified activity. By using small and precise pieces of DNA it is also possible to manipulate the actual active site of a wild-type protein in order to obtain a smaller polypeptide endowed with the same or similar activity; due to its lower size, the new polypeptide might thus be administered to a patient by different administration routes than the wild-type protein (i.e. by inhalation or transdermally or transmucosally rather than by injection) or possess other advantages such as increase yield during recombinant production of the therapeutic protein.

As will become apparent from the following detailed description and from the examples, the present method is based on a series of PCR amplification steps which are initially carried out on two non-contiguous DNA segments X1 and X2 which are thus fused together. The thus obtained polynucleotide (X1X2) is then inserted in a suitable expression vector to express the desired polypeptide in recombinant cells or transgenic animals according to known methods. The polypeptide may be a fusion polypeptide, or a polypeptide identical to a protein that is naturally produced by translation of a cDNA of polynucleotide elements X1 and X2, where X1 and X2 are exon sequences that are not adjacent in the genomic DNA. Additional chimeric polypeptides within the scope of the invention include dual-activity polypeptides, such as luteinizing hormone-follicle stimulating hormone (LH-FSH) or single polypeptide, propolypeptide, or prepropolypeptide

wherein the mature protein is LH, FSH, thyroid stimulating hormone (TSH), chorionic gonadotropin (CG), or any other active polypeptide.

In another aspect, the present invention is directed to a chimeric follicle stimulating hormone (FSH) polypeptide having FSH activity but comprising, instead of two separate polypeptide chains (designated α and β in the native molecule), a single fusion polynucleotide segment encoding the α -chain having its 3' end directly fused to the 5' end of the β -chain. The encoded chimeric polypeptide molecule is termed AB-FSH, and has been shown to possess FSH activity. This method of producing a chimeric AB-FSH protein allows for simplified expression of active follicle stimulating hormone, as the complete protein is encoded in a single vector and expressed from a single promoter. The method allows for easy purification of an active, stable AB-FSH fusion protein, free from isolated beta-FSH and/or alpha-FSH chains.

Preferably, AB-FSH is expressed with the signal sequence for the α -chain to direct secretion of the fusion protein out of the expressing cell and facilitate its subsequent purification. The signal sequence would be cleaved off in post-translational modification. Alternately, the signal sequence of the β chain can be used 5' to the α chain, or the signal sequence of the α chain can be used 5' to the β chain.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of two pieces of starting material (double-stranded polynucleotide segments) and the associated primers used in the initial PCR reactions to amplify starting materials. PFX1 is a forward PCR primer for Exon 1; PRX1 is a reverse PCR primer for Exon 1; "OH" and "HO" represent 3' terminal hydroxy groups of the double stranded DNA; PFX2 is a forward PCR primer for Exon 2; and PRX2 is a reverse PCR primer for Exon 2.

Figure 2 is a schematic representation of the PCR step to generate an intermediate PCR product in which a portion of the 5' end of X2 is added to the 3' end of the X1 to make X1UR or a portion of the 3' end of the X1 is added to the 5' end of

X2 to make DRX2. PRX1-PFX2' is a reverse PCR primer made by combining the PRX1 primer sequence with the complementary sequence of the PFX2 primer (PFX2') ; and PRX1'-PFX2 is a forward PCR primer made by combining the complementary sequence of the PRX1 primer (PRX1') with the PFX2 primer sequence.

Figure 3 is a schematic representation of the third PCR reaction step, which illustrates the steps of denaturation of the intermediate product X1UR and the X2 starting material, followed by annealing to form a mix of Species A and Species B, followed by extension in which Species A forms X1X2 by 5'-3' polymerization and Species B forms X1X2 by simultaneous 5'-3' polymerization and 5'-3' exonuclease activity. X1X2 is then further amplified in subsequent rounds of denaturation, annealing and extension. Note that an equivalent mechanism would result in the formation of the X1X2 species from X1 and DRX2 templates using primers PFX1 and PRX2.

In Figure 3, the symbol:





-  represents Taq DNA polymerase;
-  indicates the directionality of Taq DNA polymerase processivity; and
-  and  represent nucleotides released from Species B by the 5'-3' exonuclease activity of Taq DNA polymerase.

Figure 4 is a photograph of an agarose gel showing the successful PCR amplification of the X1 beta-FSH PCR product (lane 2), the X2 beta-FSH PCR product (lane 3), and the X1X2 beta-FSH PCR product (lane 4). Lane 1 is the ØX/Hinc II MK13a (HT Biotechnology Ltd., Cambridge, UK) molecular weight markers.

Figure 5 is a photograph of an agarose gel showing the successful PCR amplification of the S-FSH-B PCR product (lane 2), the glycalA RT-PCR product (lane 3), and the AB-FSH (alpha-beta-FSH) PCR product (lane 4). Lane 1 is the ØX/Hinc II MK13a (HT Biotechnology Ltd., Cambridge, UK) molecular weight markers.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.*, 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Definitions

"Amplification" of DNA as used herein denotes the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. For a description of PCR see Saiki *et al.*, Science 1988, 239:487.

The term "gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins or enzymes, which DNA sequence may or may not include regulatory DNA sequences (such as promoter sequences) and untranslated sequences (such as the 5' untranslated region, 3' untranslated region, and introns). Some genes, which are not structural genes, may be transcribed from DNA to RNA, but are not translated into an amino acid sequence.

In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of defining the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the transcribed mRNA, also known as the

sense strand or the "forward" strand). The organization of other DNA sequences relative to the particular double-stranded DNA molecule may be described herein according to the normal convention wherein sequences at the 5' end of the particular double-stranded DNA are "upstream" sequences (UR), and sequences at the 3' end of the particular double-stranded DNA are "downstream" (DR) sequences. Note however, that the present method is valid for the amplification of both the reference sense ("forward") strand and of its complementary antisense (or "reverse") strand.

In discussing the structure of a particular single stranded polynucleotide (such as an oligonucleotide PCR primer or an isolated strand of a double stranded DNA molecule), sequences are described herein according to the normal convention of defining the sequence in the 5' to 3' direction, which 5' end represents the terminal phosphate end of said single stranded polynucleotide and which 3' end represents the terminal hydroxy end of said single stranded polynucleotide. In particular, a "forward" primer is an oligonucleotide which hybridizes to the 3' end of the antisense (or "reverse") strand in order to direct 5'-3' polymerization of the complementary sense (or "forward" strand). Conversely, a "reverse" primer is an oligonucleotide which hybridizes to the 3' end of the sense (or "forward") strand in order to direct 5'-3' polymerization of the complementary antisense (or "reverse") strand.

By "expression construct", "expression vector" or "construct" is meant a nucleic acid sequence comprising a target nucleic acid sequence or sequences whose expression is desired, operably linked to sequence elements which provide for the proper transcription and translation of the target nucleic acid sequence(s) within the chosen host cells. Such sequence elements may include, for example, a promoter, a signal sequence for secretion, and polyadenylation signal. The expression construct", "expression vector" or "construct" further comprises "vector sequences". By "vector sequences" is meant any of several nucleic acid sequences established in the art which have utility in the recombinant DNA technologies of the invention to facilitate the cloning and propagation of the expression constructs including (but not limited to) plasmids, cosmids, phage vectors, viral vectors, and yeast artificial chromosomes.

The expression constructs of the invention can be introduced into a host cell, so as to transform the host and promote expression (e.g. transcription and translation) of the introduced target nucleic acid sequence.

A "promoter" or "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. The promoter may be operatively associated with other expression control sequences, including enhancer and repressor sequences.

A sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, i.e., the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

By "host cell" is meant a cell which has been transfected or transformed with one or more expression constructs of the invention. Such host cells include prokaryotic and eukaryotic cells. Preferred eukaryotic cells for use in the present invention are in vitro cultured mammalian cells, such as COS cells and CHO cells. The term host cells also encompasses transformed cells found in vivo, such as in a transgenic mammal.

By "transfection" or "transformation" is meant the process of introducing one or more of the expression constructs of the invention into a host cell by any of the methods well established in the art, including (but not limited to) microinjection,

electroporation, liposome-mediated transfection, calcium phosphate-mediated transfection, or virus-mediated transfection. A host cell into which an expression construct of the invention has been introduced by transfection or transformation is "transfected" or "transformed".

Amplification of the chimeric polynucleotide X1X2

The present inventors have discovered a method for amplifying a chimeric polynucleotide (X1X2) comprising two non-contiguous nucleotide segments of interest, X1 and X2. In one embodiment, the X1 and X2 sequences are derived from sequences that encode distinct proteins, such that the X1X2 sequence encodes a fusion protein. In another embodiment, the X1 and X2 sequences are derived from sequences that encode a single protein. For example, X1 and X2 may represent the sequences of two exons of a gene, which are not adjacent in the genomic sequence of the gene (e.g. due to separation by introns or possibly other exons or a portion of an exon), but which are adjacent in the transcribed mRNA of the gene (e.g. due to splicing to remove intervening sequences). In this case, the method of the present invention allows for the direct production of the joined X1X2 sequence, wherein X2 is immediately 3' to X1, without the necessity of isolating spliced RNA, making cDNA, or performing complex restriction digestion and ligation of genomic DNA sequences.

The two nucleotide segments of interest, X1 and X2, can be obtained from a nucleic acid mixture comprising a nucleic acid molecule which includes X1 and a nucleic acid molecule which includes X2. Alternatively, the two nucleotide segments of interest, X1 and X2, are included in the same nucleic acid molecule but they are not contiguous. For example, the nucleic acid molecule may be genomic DNA. Preferably, the nucleic acid molecules are derived from a mammal, particularly a human.

In one embodiment of the present invention, the method comprises four different PCR reactions:

In the first reaction a first nucleic acid segment X1 is made and amplified with a first primer set, which primer set comprises (i) a first primer, PFX1 (which stands for "primer forward for X1") that hybridizes to the 3' end of one strand of the X1 sequence and (ii) a second primer, PRX1 (which stands for "primer reverse for X1") that hybridizes to 3' end of the complementary strand of the X1 sequence (Figure 1). The amplified X1 PCR product may be isolated by any suitable method (as described, for example, in "Molecular Cloning: A Laboratory Manual." 2nd Edition. Sambrook, *et al.* Cold Spring Harbor Laboratory:1989, "A Practical Guide to Molecular Cloning" Perbal:1984, and "Current Protocols in Molecular Biology" Ausubel, *et al.*, eds. John Wiley & Sons:1989; commercially available kits include, e.g., the QIAquick PCR Purification Kit and the QIAEX II Gel Extraction Kit, both from Qiagen)

In the second reaction, a second nucleic acid segment X2 is made and amplified with a second primer set, which second primer set comprises (i) a second forward primer, PFX2, that hybridizes to the 3' end of one strand of the X2 sequence and (ii) a second reverse primer, PRX2, that hybridizes to the 3' end of the complementary strand of the X2 sequence. The amplified X2 product can be isolated or purified using any suitable method.

In the third PCR reaction (Figure 2), an intermediate molecule X1UR or DRX2 is made and amplified. X1UR comprises the entire first nucleic acid segment of X1 and a relatively small 5' segment of X2, wherein the 3' end of X1 is fused to the 5' segment of X2. DRX2 comprises the entire first nucleic acid segment of X2 and a relatively small 3' segment of X1, wherein the 5' end of X2 is fused to the 3' segment of X1. This step is carried out with a third primer set. For X1UR, the third primer set comprises (i) PFX1 and (ii) a fusion primer PRX1-PFX2', which fusion primer has the nucleotide sequence of PRX1 followed immediately at its 3' end by the sequence of the complement of PFX2 (designated as PFX2'). For DRX2, the third primer set comprises (i) a fusion primer PRX1'-PFX2, which fusion primer has the nucleotide sequence of the complement of PRX1 (designated as PRX1') attached

to the 5' end of the forward primer PFX2, and (ii) the primer PRX2. Either intermediate can be isolated or purified using any suitable method.

In the fourth reaction (Figure 3), the desired polynucleotide X1X2 is finally made and amplified. This PCR reaction relies on the fact that for many commercially available sources of Taq DNA polymerase, the Taq enzyme possesses both 5'-3' DNA polymerase activity and 5'-3' exonuclease activity at certain temperatures.

In one embodiment of this fourth reaction, the following reagents are used: the X1UR amplified product, the X2 amplified product and the primers PFX1 and PRX2. In this reaction, both X1UR and X2 serve as templates for PCR using primers PFX1 and PRX2. The templates are annealed to form two species of significance here: Species A and Species B.

Species A consists of the forward (5'-3') strand of X1UR and the reverse (3'-5') strand of X2, with the two strands annealed via the complementary X2-derived sequences of the UR region of X1UR and the 3' end of the reverse strand of X2. Although neither primer binds to Species A, the Taq polymerase will extend the 3' ends of the annealed overlap region to produce the double stranded species X1X2.

Species B consists of the reverse (3'-5') strand of X1UR and the forward (5'-3') strand of X2, with the two strands annealed via the complementary X2-derived sequences of the UR region of X1UR and the 5' end of the forward strand of X2. Both primers bind to this species. The 3' ends of the annealed primers are then extended by the 5'-3' polymerase activity of the Taq polymerase. Both strands of the final X1X2 product will be intact, because as each primer is extended on its template in the 5'-3 direction, the original annealed strand (reverse of X1UR or forward of X2) is removed by the 5'-3' exonuclease activity of the Taq polymerase. In other words, there will be no "nick" in the strand where the extension product of PRX2 "meets" the 5' end of the reverse strand of X1UR because the latter will be removed by the Taq enzyme, while the (newly synthesized) forward strand of X1X2 will serve as a template for the remainder of the extension product of PRX2 to be built. The same process is at work to result in the full forward strand of X1X2: when the extension

product of primer PRX1 "meets" the 5' end of the forward strand of X2, this strand is removed by the Taq enzyme, while the (newly synthesized) reverse strand of X1X2 will serve as a template for the remainder of the extension product of PFX1 to be built.

In an alternate embodiment of this fourth reaction, the following reagents are used: the DRX2 amplified product, the X1 amplified product, and the primers PFX1 and PRX2. In this reaction, both DRX2 and X1 serve as templates for PCR using primers PFX1 and PRX2. This version of the fourth PCR reaction, wherein annealing to form Species A and Species B is mediated by the complementary X1-sequences of X1 and the DR region of DRX2, is analogous to the embodiment described immediately above and also encompassed by the present specification.

In yet another embodiment of this fourth reaction, the following reagents are used: the X1UR amplified product, the DRX2 amplified product, and the primers PFX1 and PRX2. In this reaction, both X1UR and DRX2 serve as templates for PCR using primers PFX1 and PRX2. This version of the fourth PCR reaction, wherein annealing to form Species A and Species B is mediated both by the complementary X2-sequences of the UR region of X1UR and the 5' end of the forward strand of X2 and by the the complementary X1-sequences of X1 and the DR region of DRX2, is analogous to the embodiment described immediately above and also encompassed by the present specification.

It will be understood that for extension of Species A no oligonucleotide primers are necessary. Therefore, the forgoing directions to employ a polymerase that also acts as an exonuclease pertain to amplification of Species B. In practice, however, both extension of Species A and Species B will take place in the same reaction mixture for reasons of efficiency and convenience, so the same enzyme will be used for both.

In other embodiments of the present invention, the method comprises fewer than four different PCR reactions.

For example, the present method does not require that both (or all) of the starting DNA segments X1 and X2 be PCR amplified or isolated. Therefore, the intermediate products X1UR and/or DRX2 may be generated directly from the primary template sequences (e.g. genomic DNA or cDNA) without first performing a PCR to generate X1 or X2. For example, the primer PFX1 and the hybrid primer PRX1-PFX2' may be used to amplify X1UR directly from genomic DNA. Similarly, the primer PRX2 and the hybrid primer PRX1'-PFX2 may be used to amplify DRX2 directly from genomic DNA. Starting from amplified or isolated X1 and X2 sequences, however, makes the subsequent amplification steps more efficient.

The segment X2 should be amplified or isolated when it is combined with X1UR to assemble the chimeric DNA molecule X1X2. Similarly, the segment X1 should be amplified or isolated when it is combined with DRX2 to assemble the chimeric DNA molecule X1X2.

According to the preferred embodiment (hereinafter referred to as "Strategy #2"), the present invention is directed to a method for making a polynucleotide (X1X2) comprising two nucleotide segments of interest, X1 and X2, wherein X2 in X1X2 is immediately 3' to X1, from a nucleic acid molecule including X1 and the same or a different nucleic acid molecule including X2, wherein if X1 and X2 originate on the same molecule, they are not contiguous, the method comprising:

(a) amplifying a first double stranded nucleic acid segment X1, which segment comprises a sense and an antisense nucleic acid strand, with a first primer set, which primer set comprises (i) a forward primer, PFX1, which hybridizes to the 3' end of the antisense strand of X1 and (ii) a reverse primer, PRX1, which hybridizes to the 3' end of the sense strand of X1;

(b) amplifying a second double stranded nucleic acid segment, X2, which segment comprises a sense and an antisense nucleic acid strand, with a second primer set, which second primer set comprises (i) a forward primer, PFX2, which hybridizes to the 3' end of the antisense strand of X2 and (ii) a reverse primer, PRX2, which hybridizes to the 3' end of the sense strand of X2;

(c) isolating the X1 and X2 products of steps (a) and (b)

(d) performing PCR in a single reaction vessel, said vessel comprising the isolated X1 and X2 products of step (c) in stoichiometric amounts and primers PFX1, PRX2, and a fusion primer, which fusion primer has the nucleotide sequence of PRX1 preceded at its 5' end by the sequence of the complement of PFX2, said PFX2 complement termed PFX2', wherein PCR performed in this single vessel results in amplification of an intermediate double stranded polynucleotide X1UR, which intermediate comprises a sense and an antisense nucleic acid strand, and which comprises the double stranded nucleic acid segment X1 and a 5' double stranded nucleic acid segment of X2, wherein the 3' end of X1 is fused to the 5' segment of X2, and wherein said reaction also results in amplification of the X1UR intermediate to make X1X2 by denaturing and annealing X1UR and X2 to form annealed species and then extending and amplifying said annealed species using DNA polymerase possessing both 5'-3' polymerase activity and 5'-3' exonuclease activity and primers PFX1 and PRX2.

Alternatively, step (d) can be carried out by performing PCR in a single reaction vessel, said vessel comprising the isolated X1 and X2 products of step (c) in stoichiometric amounts and primers PRX1, PFX2, and a fusion primer, which fusion primer has the nucleotide sequence of PFX2 preceded at its 5' end by the sequence of the complement of PRX1, said PRX1 complement termed PRX1', wherein PCR performed in this single vessel results in amplification of an intermediate double stranded polynucleotide DRX2, which intermediate comprises a sense and an antisense nucleic acid strand, and which comprises the double stranded nucleic acid segment X2 and a 3' double stranded nucleic acid segment of X1, wherein the 5' end of X2 is fused to the 3' segment of X1, and said reaction also results in amplification of the DRX2 intermediate to make X1X2 by denaturing and annealing DRX2 and X1 to form annealed species and then extending and amplifying said annealed species using DNA polymerase possessing both 5'-3' polymerase activity and 5'-3' exonuclease activity and primers PRX1 and PFX2.

It will be understood that the method of the present invention may be used to further attach additional sequences (X3) to the chimeric molecule X1X2. For example, where DNA pieces X1X2 and X3 are to be assembled into the polynucleotide X1X2X3, the segment X1X2 would also have an extension complementing the 5' end of X3 if the UR approach is used (as apposed to the DR approach, where X1X2 would be joined to the segment X3, which has an extension complementing the 3' end of X1X2).

The foregoing methods are not limited to making nucleic acid sequences that encode chimeric proteins, or to assembling nucleic acid sequences that encode multi-exon proteins from genomic source DNA. Any two or more polynucleotide segments can be fused, including restriction fragments from one or more DNA molecules. The foregoing method can be easily adapted to produce polynucleotides comprising three or more noncontiguous or heterologous nucleic acid molecules, or segments of such molecules

Suitable Taq polymerases that have the combined polymerase/exonuclease activity are commercially available. See, e.g., *Pfu* DNA polymerase (# M7741 or # M7745); *Tth* DNA polymerase (# M2101 or # M2105); *Tfi* DNA polymerase (# M1945 or # M1945), all available from Promega Corporation, Madison WI, USA.

The newly amplified polynucleotide of interest X1X2 can thus be isolated by means of standard methodologies and can be inserted into a suitable expression system to provide expression of the corresponding fusion polypeptide.

Expression constructs

The expression constructs of the invention contain the amplified polynucleotide sequence operably linked to elements necessary for proper transcription and translation of the amplified sequences within the chosen host cells, including a promoter, a translation initiation signal ("start" codon), a translation termination signal ("stop" codon) and a polyadenylation signal. The expression constructs may comprise additional sequences that modify expression of the

amplified sequences, including internal ribosome entry sites (IRES), enhancers, response elements, suppressors, signal sequences, and the like.

The promoter sequences may be endogenous or heterologous to the host cell, and may provide ubiquitous (*i.e.*, expression occurs in the absence of an apparent external stimulus and is not cell-type specific) or tissue-specific (also known as cell-type specific) expression.

Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patents No. 5,385,839 and No. 5,168,062), the SV40 early promoter region (Benoist and Chambon, *Nature* 1981; 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, *Cell* 1980, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner *et al.* *Proc. Natl. Acad. Sci. USA* 1981;82:3567-71), and the herpes simplex virus LAT promoter (Wolfe, *et al.* *Nature Genetics* 1992;1:379-384), the regulatory sequences of the metallothionein gene (Brinster *et al.*, *Nature* 1982, 296:39-42); prokaryotic expression vectors such as the beta-lactamase promoter (Villa-Komaroff, *et al.*, *Proc. Natl. Acad. Sci. USA* 1978, 75:3727-3731), or the tac promoter (DeBoer, *et al.*, *Proc. Natl. Acad. Sci. USA* 1983, 80:21-25); see also "Useful proteins from recombinant bacteria" in *Scientific American* 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; the human beta-actin promoter (Gunning, *et al.* *Proc. Natl. Acad. Sci. USA* 1987;84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR; Klessig, *et al.* *Mol. Cell Biol.* 1984;4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR; Weiss, *et al.* *RNA Tumor Viruses*. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.:1985).

The expression constructs further comprise vector sequences that facilitate the cloning and propagation of the expression constructs. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or

expression in a variety of eukaryotic and prokaryotic host cells. Standard vectors useful in the current invention are well known in the art and include (but are not limited to) plasmids, cosmids, phage vectors, viral vectors, and yeast artificial chromosomes. The vector sequences may contain a replication origin for propagation in *E. coli*; the SV40 origin of replication; an ampicillin, neomycin, or puromycin resistance gene for selection in host cells; and/or genes (*e.g.*, dihydrofolate reductase gene) that amplify the dominant selectable marker plus the gene of interest. Prolonged expression of the encoded target-reporter fusion in *in vitro* cell culture may be achieved by the use of vectors sequences that allow for autonomous replication of an extrachromosomal construct in mammalian host cells (*e.g.*, EBNA-1 and oriP from the Epstein-Barr virus).

For example, a plasmid is a common type of vector. A plasmid is generally a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional foreign DNA and which can readily be introduced into a suitable host cell. A plasmid vector generally has one or more unique restriction sites suitable for inserting foreign DNA. Examples of plasmids that may be used for expression in prokaryotic cells include, but are not limited to, pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids, and pUC-derived plasmids.

A number of vectors exist for expression in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, *e.g.*, Broach, *et al.* "Experimental Manipulation of Gene Expression." ed. M. Inouye (Academic Press:1983)). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid.

A number of expression vectors exist for expression in mammalian cells. Many of these vectors contain prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription regulatory sequences

that cause expression in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo, and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified by the addition of sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) may be used for transient expression of proteins in eukaryotic cells. A baculovirus expression system (see, e.g., "Current Protocols in Molecular Biology." eds. Ausubel *et al.* (John Wiley & Sons:1992)) may also be used. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the β -gal containing pBlueBac III).

For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see "Molecular Cloning A Laboratory Manual. 2nd Edition." Sambrook, *et al.* (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

The expression constructs of the invention may be transfected or transformed into eukaryotic or prokaryotic host cells *in vitro*. Preferred *in vitro* host cells are mammalian cell lines, such as COS cells and CHO cells. Protocols for *in vitro* culture of mammalian cells are well established in the art [see for example, *Animal Cell Culture: A Practical Approach 3rd Edition*. J. Masters, ed. Oxford University Press and *Basic Cell Culture 2nd Edition*. Davis, J.M. ed. Oxford University Press (2002)]. Techniques for transfection and transformation are well established in the art and may include electroporation, microinjection, liposome-mediated transfection, calcium phosphate-mediated transfection, or virus-mediated transfection [see for example, *Artificial self-assembling systems for gene delivery*. Felgner, *et al.*, eds. Oxford University Press (1996); Lebkowski, *et al.* Mol Cell Biol 1988 8(10):3988-

3996; "Molecular Cloning: A Laboratory Manual." 2nd Sambrook, *et al.* Cold Spring Harbor Laboratory:1989; and "Current Protocols in Molecular Biology" Ausubel, *et al.*, eds. John Wiley & Sons:1989).

Production of biologically active chimeric proteins

According to a preferred aspect of the invention, the present method is used to produce a polynucleotide X1X2 which encodes a biologically active polypeptide E1E2, wherein E1 is the polypeptide sequence encoded by X1 and E2 is the polypeptide sequence encoded by X2.

According to another aspect of the present invention the two nucleotide segments X1 and X2 are two exons which are both present in the human genome and wherein each encodes one of two fragments of a biologically active protein.

In one example, X1 and X2 encode the two exons of the beta subunit of FSH.

In another example, X1 and X2 encode two subunits of a multi-subunit protein, the alpha-subunit and beta-subunit of FSH. Therefore, according to a further aspect of the present invention the polynucleotide (X1X2) encodes a single polypeptide having the activity of chorionic gonadotropin (CG), luteinizing hormone (LH), follicle stimulating hormone (FSH) or thyroid stimulating hormone (TSH); or encodes a single polypeptide having the activity of more than one of these hormones (e.g., activity of both LH and FSH).

Alternatively, the polynucleotide (X1X2) may encode a pre-protein E1E2, wherein the segment E1 represents a fusion partner, as for instance a signal sequence, and E2 is a biologically active mature polypeptide. The signal sequence may be the natural signal sequence of E2 or a different signal sequence. The objective would be to facilitate secretion of the mature E2.

For example we can produce the β subunit of FSH consisting of segments X1 and X2. Alternatively, we can produce any other chimeric polypeptide by using heterologous segments of DNA fused together. So the chimeric protein will have properties of both the constituent polypeptides. New and unique molecules can thus be generated.

As it will be apparent from the examples, which relate to the expression of the beta-subunit of hFSH and to the expression of a chimeric protein containing both the alpha and beta subunits of FSH (termed AB-FSH), the method according to the present invention can be carried out using any two (or more) non contiguous DNA segments. If additional segments are used, additional PCR steps will be necessary, as a person of ordinary skill will readily appreciate.

Examples

The present invention is next described by means of the following examples. However, the use of these and other examples anywhere in the specification is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified form. Likewise, the invention is not limited to any particular preferred embodiments described herein. Indeed, many modifications and variations of the invention may be apparent to those skilled in the art upon reading this specification, and can be made without departing from its spirit and scope. The invention is therefore to be limited only by the terms of the appended claims, along with the full scope of equivalents to which the claims are entitled.

Example 1: Assembly of beta-FSH expression constructs

using a novel PCR methodology

The sequence of the genomic DNA encoding the β subunit of human FSH is at Genbank Accession # AH003599. The cDNA sequence of the β subunit of human FSH is SEQ ID NO:1. The signal sequence is depicted as SEQ ID NO: 2. Exon 1 has the sequence SEQ ID NO: 3; Exon 2 has the sequence SEQ ID NO: 4. The corresponding amino acid sequence of human β -FSH is at SEQ ID NO: 5. It is to be noted that β FSH exists in various polymorphs of which the present example produces only one. However, any other polymorph could have been easily produced by the present method.

A PCR amplification of beta-FSH encoding sequences

- 1. Isolation and amplification of two DNA segments each encoding one of two exons of beta-FSH*

Human genomic DNA was extracted from 50µl of total blood using the Nucleo Spin Blood Quick Pure kit (Macherey-Nagel GmbH& Co.). The isolated DNA was then dissolved in 100 µl of TB Buffer (DNA solution). 10 µl of this DNA solution was used as template for two independent PCR reactions (5µl genomic DNA solution per PCR reaction).

The first PCR reaction was performed using primers specific for exon 1 of beta-FSH. The forward primer PFX1 with the sequence (SEQ ID NO: 7) 5'-ATG AAG ACA CTC CAG TTT TTC TTC C-3' corresponds to a segment from position 40 to position 64 in SEQ ID NO: 1. The reverse primer PRX1 with the sequence (SEQ ID NO: 8) 5'-CCT GGT GTA GCA GTA GCC AGC-3' corresponds to the complement of the segment from position 198 to position 178 of SEQ ID NO: 1. This PCR reaction amplifies the product X1, as illustrated at Figure 1, left scheme.

The second PCR reaction was performed using primers specific for exon 2 of beta-FSH. The forward primer PFX2 with the sequence (SEQ ID NO: 9) 5'-GAT CTG GTG TAT AAG GAC CCA-3' corresponds to the segment from position 199 to position 219 of SEQ ID NO: 1. The reverse primer PRX2 with the sequence (SEQ ID NO: 10) 5'-TTA TTC TTT CAT TTC ACC AAA GG-3' corresponds to the complement of the segment from position 429 to position 407 of SEQ ID NO: 1. This PCR reaction amplifies the product X2, as illustrated at Figure 1, left scheme.

Each independent PCR reaction contained 5 µl human genomic DNA solution as the template, and the following additional reagents:

1. 10 µl 10X PCR Buffer (Thermophilic DNA Polymerase 10 x Buffer, magnesium Free M190G, Promega Madison WI, USA)
2. 10 µl 25 mmol MgCl₂
3. 5 µl dNTPs stock solution containing 10 µmol each of dATP, dTTP, dCTP, and dGTP.
4. 1 µl of 100pmol/µl forward primer stock dilution
5. 1 µl of 100pmol/µl reverse primer stock dilution

6. 2 μ l TAQ (5 U/ μ l) (Taq DNA Polymerase in Storage Buffer A, Cat No. Mi865, Promega Madison WI, USA)
7. 65 μ l H₂O.

Cycling parameters were as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification with each cycle consisting of 30 sec at 94°C, followed by 30 sec at 50°C, and then 1 min at 72°C; and a final extension for 5 min at 72°C. Cycling was performed using a PTC-100™ programmable Thermal Controller (MJ Research Inc, Watertown, Mass, USA).

Amplification of the X1 and X2 products (beta-FSH exon 1 and exon 2 segments, respectively) by each PCR was confirmed by agarose gel (2% in TAE buffer) electrophoresis for 30 min at 125mA using the DNA Molecular Weight Marker ØX/Hinc II MK13a (HT Biotechnology Ltd., Cambridge, UK) as control molecular weight markers. The results are shown in Figure 4 (see Lanes 2 and 3). The X1 and X2 products of the PCR reaction were then purified using the PCR Clean-up Kit (Nucleospin Extract. Cat No. 740590.50).

2. *PCR amplification of the XIUR intermediate product:*

A PCR reaction was performed on the X1 product (prepared as describe above) as template, using the exon 1 forward primer PFX1 (SEQ ID NO: 7) and a hybrid reverse primer PRX1-PFX2' having the sequence (SEQ ID NO: 11): 5'-TGG GTC CTT ATA CAC CAG ATC CCT GGT GTA GCA GTA GCC AGC-3', where the sequence of PRX1 is italicized and the reverse complement of the sequence of PFX2 is underlined.

The PCR reaction mixture contained 1 μ l X1 PCR product (exon 1 of beta-FSH: SEQ ID NO: 3) solution as the template, and the additional reagents listed above. Cycling parameters were as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification with each cycle consisting of 30 sec at 94°C, followed by 1 min at 50°C, and then 2 min at 68°C; and a final extension for 10 min at 72°C. Cycling was performed using a PTC-100™ programmable Thermal Controller (MJ

Research Inc, Watertown, Mass, USA). This reaction is illustrated at Figure 2, top scheme.

Amplification of the X1UR intermediate product by PCR was confirmed by agarose gel (2% in TAE buffer) electrophoresis for 30 min at 125mA using the DNA Molecular Weight Marker ØX/Hinc II MK13a (HT Biotechnology Ltd., Cambridge, UK) as control molecular weight markers. The X1UR PCR product was then purified using the PCR Clean-up Kit (Nucleospin Extract, Cat No. 740590.50) to yield pure DNA in a final volume of 50µl. Purity and size of the DNA was confirmed by agarose (2% in TAE buffer) electrophoresis for 30 min at 125mA using the DNA Molecular Weight Marker ØX/Hinc II MK13a (HT Biotechnology Ltd., Cambridge, UK) as control molecular weight markers.

The intermediate X1UR beta-FSH PCR product (SEQ ID NO: 13) consists of the X1 (beta-FSH exon 1; SEQ ID NO:3) sequence extended at its 3' end by the first 21 base pairs of SEQ ID NO:4 (the beta-FSH exon X2 sequence).

3. *PCR amplification of the X1X2 (Exon 1-Exon 2 beta-FSH) product*

Two different strategies were employed to amplify the product X1X2.

Strategy #1:

In the first strategy, illustrated at Figure 3, a PCR reaction was performed using the exon 1 forward primer PFX1 (SEQ ID NO: 7) and a the exon 2 reverse primer PRX2 (SEQ ID NO: 10). The earlier amplified products X1UR and X2 were the templates for this reaction. This PCR reaction contained the following reagents:

1. 10 µl 10X PCR Buffer (Thermophilic DNA Polymerase 10 x Buffer, magnesium Free M190G, Promega Madison WI, USA)
2. 10 µl 25 mmol MgCl₂
3. 5 µl dNTPs stock solution containing 10 µmol each of dATP, dTTP, dCTP, and dGTP.
4. 1 µl X1UR intermediate PCR product (SEQ ID NO: 13)
5. 1 µl X2 PCR product (exon 2 of beta-FSH: SEQ ID NO: 4)

6. 1 μ l of 100pmol/ μ l PFX1 (beta-FSH exon 1 forward primer (SEQ ID NO: 7)) stock dilution
7. 1 μ l of 100pmol/ μ l PRX2 (beta-FSH exon 2 reverse primer (SEQ ID NO: 10)) stock dilution
8. 2 μ l TAQ (5 U/ μ l) (Taq DNA Polymerase in Storage Buffer A, Cat No. Mi865, Promega Madison WI, USA)
9. 65 μ l H₂O

Cycling parameters were as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification with each cycle consisting of 30 sec at 94°C, followed by 1 min at 50°C, and then 2 min at 68°C; and a final extension for 10 min at 72°C. Cycling was performed using a PTC-100TM programmable Thermal Controller (MJ Research Inc, Watertown, Mass, USA).

Amplification of the X1X2 product by this PCR reaction was confirmed by agarose (2% in TAE buffer) electrophoresis performed as described above. The results are shown in Figure 4 (see Lane 4). The product of this PCR reaction was then purified using the PCR Clean-up Kit (Nucleospin Extract, Cat No. 740590.50) to yield pure DNA in a final volume of 50 μ l. Purity and size of the DNA was confirmed by agarose (2% in TAE buffer) electrophoresis performed as described above.

The size of the product was determined by agarose gel electrophoresis (performed as described above) to be about 390 bp. The sequence of this product was confirmed to be the full length coding sequence for beta-FSH, X1X2 (SEQ ID NO: 6; see Genbank Accession # NM_000510), by conventional sequence analysis using the primer PFX1 (beta-FSH exon 1 forward primer (SEQ ID NO: 7)).

Strategy #2:

In an alternate strategy the final X1X2 PCR product was generated by a single three primer PCR using the X1 and X2 products as template. This strategy shortened the cloning process by one PCR step. In this strategy, the primers PRX2, PFX1, and the hybrid reverse primer PRX1-PFX2' were used on the X1 and X2 products as

template, thereby amplifying first the X1UR intermediate and then the X1X2 final product in a single PCR reaction. The PCR reaction contained the following reagents:

1. 10 µl 10X PCR Buffer (Thermophilic DNA Polymerase 10 x Buffer, magnesium Free M190G, Promega Madison WI, USA)
2. 10 µl 25 mmol MgCl₂
3. 5 µl dNTPs stock solution containing 10 µmol each of dATP, dTTP, dCTP, and dGTP.
4. 2 µl X1 PCR product (SEQ ID NO: 3)
5. 2 µl X2 PCR product (exon 2 of beta-FSH: SEQ ID NO: 4)
6. 1 µl of 100pmol/µl PRX2 (beta-FSH exon 2 reverse primer (SEQ ID NO: 10)) stock dilution
7. 1 µl of 100pmol/µl forward primer PFX1 (beta-FSH exon 1 forward primer (SEQ ID NO: 7) stock dilution
8. 1 µl of 100pmol/µl hybrid reverse primer PRX1-PFX2' (SEQ ID NO: 11) stock dilution
9. 2 µl TAQ (5 U/µl) (Taq DNA Polymerase in Storage Buffer A, Cat No. Mi865, Promega Madison WI, USA)
10. 65 µl H₂O

Cycling parameters were as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification with each cycle consisting of 30 sec at 94°C, followed by 1 min at 50°C, and then 2 min at 68°C; and a final extension for 10 min at 72°C. Cycling was performed using a PTC-100TM programmable Thermal Controller (MJ Research Inc, Watertown, Mass, USA).

Amplification of the X1X2 product by this PCR reaction was confirmed by agarose (2% in TAE buffer) electrophoresis performed as described above. The product of this PCR reaction was then purified using the PCR Clean-up Kit (Nucleospin Extract. Cat No. 740590.50) to yield pure DNA in a final volume of

50µl. Purity and size of the DNA was confirmed by agarose (2% in TAE buffer) electrophoresis performed as described above.

The size of the product was determined by agarose gel electrophoresis (performed as described above) to be about 390 bp. The sequence of this product was confirmed to be the full length coding sequence for beta-FSH, X1X2 (SEQ ID NO: 6; see Genbank Accession # NM_000510), by conventional sequence analysis using the primer PFX1 (beta-FSH exon 1 forward primer (SEQ ID NO: 7)).

Though the result was the same using both PCR strategies, we used the X1X2 product from the Strategy #1 PCR reaction because it yielded a higher quantity and purity of product DNA. However, the second approach is also viable, and has the advantage of proceeding very fast, although it requires stoichiometric amounts of the templates.

4. *Addition of Shine-Delgarno and Kozak sequences to the X1X2 (Exon 1-Exon 2 beta-FSH) product*

A 17 nucleotide sequence was added to the 5' end of the X1X2 (Exon1-Exon2 beta-FSH) PCR product (SEQ ID NO: 6), in order to create Shine-Delgarno and Kozak consensus sequences (SDK), which direct translation of the expressed SDK-X1X2 transcript. The sequence was added by performing a PCR reaction on the X1X2 PCR product using the primer PRX2, the beta-FSH exon 2 reverse primer (SEQ ID NO: 10); and a new forward primer SDK-PFX1 with the sequence 5'-TCG AAG GAG ATA GAA CCA TGA AGA CAC TCC AGT TTT TCT TCC-3' (SEQ ID NO: 12), where the Shine-Delgarno and Kozak consensus sequences are underlined, the sequence of the exon 1 forward primer PFX1 (SEQ ID NO: 7) is in italics, and the "start" codon for translation initiation is in boldface. The PCR reaction contained 1 µl X1X2 (Exon1-Exon2 beta-FSH) PCR product as the template, and the following additional reagents:

1. 10 µl 10X PCR Buffer (Thermophilic DNA Polymerase 10 x Buffer, magnesium Free M190G, Promega Madison WI, USA)

2. 10 μ l 25 mmol $MgCl_2$
3. 5 μ l dNTPs stock solution containing 10 μ mol each of dATP, dTTP, dCTP, and dGTP.
4. 1 μ l of 100pmol/ μ l SDK-PFX1 primer (SEQ ID NO: 12) stock dilution
5. 1 μ l of 100pmol/ μ l PRX2 (beta-FSH exon 2 reverse primer (SEQ ID NO:10)) stock dilution
6. 2 μ l TAQ (5 U/ μ l) (Taq DNA Polymerase in Storage Buffer A, Cat No. Mi865, Promega Madison WI, USA)
7. 65 μ l H_2O .

Cycling parameters were as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification with each cycle consisting of 30 sec at 94°C, followed by 1 min at 50°C, and then 2 min at 68°C; and a final extension for 10 min at 72°C.

Amplification of the SDK-X1X2 product by this PCR reaction was confirmed by agarose (2% in TAE buffer) electrophoresis performed as described above. The product of this PCR reaction was then purified using the PCR Clean-up Kit (Nucleospin Extract, Cat No. 740590.50) to yield pure DNA in a final volume of 50 μ l. Purity and size of the DNA was confirmed by agarose (2% in TAE buffer) electrophoresis performed as described above.

B. Assembly of beta-FSH expression construct

The SDK-X1X2 PCR product was cloned into the pTARGET™ expression vector (Promega) according to manufacturer's instructions (pTARGET™ Mammalian Expression System Technical Manual). This system is based upon ligation directed by annealing of the 3' adenine overhangs on each end of a PCR product (introduced as a natural consequence of Taq polymerase activity) with 5' thymine overhangs on a linearized pTARGET™ expression vector. The ligated pTARGET™ vector was transformed into *E.coli* DH5 α .

Transformed *E.coli* DH5 α were cultured on solid medium for 24 hours, and then 10 bacterial colonies from each transformation were used to inoculate individual

liquid medium cultures. Plasmid DNA was extracted from cultured liquid medium cultures using the JET Quick Plasmid Miniprep Spin Kit/50 (Genomed GmbH, Wielandstr Bad Oeynhausen) according to manufacturer's instructions.

Isolated plasmid DNA was then checked for incorporation of the SDK-X1X2 beta-FSH insert by PCR. In this PCR, primers PFX1 (beta-FSH exon 1 forward primer (SEQ ID NO: 7)) and PRX2 (beta-FSH exon 2 reverse primer (SEQ ID NO: 10)) were used to amplify 1µl of the purified plasmid DNA template. Successful amplification of the X1X2 fragment confirmed successful cloning of the beta-FSH sequences into the pTARGET vector. The resulting expression construct was named pTPKBFSH (pTARGET containing the SDK-X1X2 PCR product insert).

Example 2: Expression of beta-FSH in mammalian cell culture

The pTPKBFSH (pTarget containing the SDK-X1X2 beta-FSH insert) expression construct was used for the expression of beta-FSH protein in COS-7L (Invitrogen, catalog No. 11622016), and CHO-S (Invitrogen, catalog No. 11619012) cell lines. The pTPKBFSH construct was transfected into the various cell lines using the LIPOFECTAMINE 2000 (Invitrogen) transfection reagent according to manufacturer's instructions. The expression vector pSV-β-Galactosidase (Promega) was included as a transfection control.

Following transfection, the cells were cultured in a selective medium containing 1000µg/ml Geneticine for 7 days. Geneticine-resistant cells were transferred in a new culture medium containing Geneticine at 200µg/ml, and cultured to a cell density of 10^6 cells/ml. The supernatant of this culture was then tested for the presence of the beta-FSH subunit using the Granulosa Cell Aromatase Bioassay (GAB assay) method for Follicle Stimulating Hormone (Dahl et al. Methods Enzymol 1989;168:414-422) for detecting bioactive human beta-FSH. This assay quantitates FSH activity based upon stimulation of aromatase activity of granulosa cells, where aromatase activity is measured by radioimmunoassay quantitation of the production of estrogen from an androstenedione precursor. Thus this assay is a

functional assay which quantifies the amount of biologically active beta-FSH protein in a test sample.

To derive Granulosa Cells for the GAB assay, intact female Spague-Dawley rats (21-22 days old) were implanted with silastic capsules (10 mm) containing approximately 10 mg diethylstilbestrol (DES) to stimulate granulosa cell proliferation. Four days later, the animals were sacrificed, and the ovaries removed and decapsulated. Follicles of the decapsulated ovaries were punctured with 27-gauge hypodermic needles, and the granulosa cells removed into McCoy's 5a medium (Gibco), supplemented with penicillin/streptomycin (100 U/mL of each) and 2mM L-glutamine. The cells were pelleted by low speed centrifugation for 5 min, and then washed with fresh medium. Cell viability was estimated by trypan blue staining of a cell aliquot, followed by cell counting using a hemacytometer. The cells were then diluted in medium to a final volume of ~2000-2400 viable cells/ ml.

On the day of the assay, fresh GAB assay medium was prepared. The GAB assay medium was: McCoy's 5a medium (Gibco), supplemented with penicillin/streptomycin (100 U/mL of each) and 2mM L-glutamine, plus 1.25 μ M androstenedione (Sigma), 0.125 μ M diethylstilbestrol (Sigma); 37.5 ng/mL human chorionic gonadotropin (Sigma: catalog # C-0434), 0.156 mM 1-methyl-3-isobutylxanthine (Sigma), and 1.25 μ g/ml insulin (Sigma: catalog # I-1507).

Then 400 μ l of GAB assay medium and 60 μ L of granulosa cell suspension (~50,000-80,000 viable cells) were added to each well of a 24-well plate. Next, 40 μ L of pTPKBFSH-transfected COS or CHO cell supernatant or of various concentrations of positive control recombinant FSH (Organon) were added to each well. The plates were then cultured for 3 days at 37°C in a humidified 5% CO₂ incubator. The supernatant from each well was then harvested, and 10-20 μ l of the supernatant assayed for estrogen levels by radioimmunoassay. Estrogen radioimmunoassay was performed using the Spectria RIA kit for estradiol measurement (Orion Diagnostic, Finland), according to the manufacturer's instructions.

For this assay, various concentrations of the positive control recombinant FSH (Organon) were tested and used to calculate a titration curve for FSH activity (recombinant FSH concentration in milliUnits per milliliter, mU/ml, versus estrogen radioimmunoassay values, see Table 1). This control curve was then used to interpolate the amount of beta-FSH activity in the supernatant of pTPKBFSH-transfected COS or CHO cells.

When a 1/1000 dilution of the supernatant from pTPKBFSH-transfected cells was tested in the granulosa assay, 348 pg/ml of estradiol was produced. This value is roughly equivalent to that seen with 25mU/ml of control recombinant FSH. After correcting for the dilution factor of the tested supernatant, this result indicates that the pTPKBFSH-transfected cell lines produce bioactive beta-FSH at a quantity of about 25 Units/ml.

Table 1: Calibration curve values for FSH activity

Concentration of recombinant FSH tested (mU/mL*)	25	50	100	200	400
Concentration of estradiol produced (pg/ml)	322	710	1900	3400	4100

* According to the manufacturer (Organon), recombinant FSH shows an activity profile of approximately 8000 Units per mg of total recombinant protein.

Example 3: Production and Expression of Chimeric DNA for AB FSH

The human FSH hormone protein is multi-subunit protein composed of an alpha subunit plus a beta subunit. The complete cDNA sequence for the alpha subunit is SEQ ID NO: 14 (Genbank Accession number NM_000735). This example describes the production of an active human FSH protein in which both subunits of the hormone are contained in a single protein chain.

A. Beta-FSH subunit encoding nucleic acid sequence

The beta-FSH subunit encoding sequence (X1X2; SEQ ID NO: 6) was prepared as described in Example 1.

B. Alpha-FSH subunit encoding nucleic acid sequence

mRNA was isolated from human placental tissue using the Oligotex Direct mRNA Mini Kit (Qiagen, Cat. No. 72022) according to manufacturer's instructions. 5 µl of the isolated mRNA was used as template in a reverse transcriptase-polymerase chain reaction (RT-PCR), using the primers HCG-SENT (5'-ATG GAT TAC TAC AGA AAA TAT GCA GCT ATC-3'; SEQ ID NO: 15) and HCG-ANTISENT (5'-TTA AGA TTT GTG ATA ATA ACA AGT ACT GCA G-3'; SEQ ID NO: 16). This RT-PCR reaction was performed using the RT-PCR Kit (Gibco) according to manufacture's instructions. The product of this RT-PCR was named glycalA, and confirmed to represent the complete coding sequence of human alpha-FSH (SEQ ID NO: 17) by 2% agarose gel electrophoresis (performed as described above; see Figure 5, lane 3) and sequencing performed using the primers HCG-SENT and HCG-ANTISENT.

The glycalA alpha-FSH RT-PCR product was then cloned into the pLenti6/V5 D-TOPO vector using the pLenti6/V5 Directional TOPO cloning kit according to manufacturer's instructions (Invitrogen K4955-10). To facilitate this cloning, the nucleotide sequence CACC was added to the 5' end of the glycalA RT-PCR product via PCR using the primers HCG-SENTCACC (5'-CAC CAT GGA TTA CTA CAG AAA ATA TGC AGC TAT C-3' SEQ ID NO: 18) and HCG-ANTISENT (SEQ ID NO: 16). This PCR contained 1µl glycalA (alpha-FSH) RT-PCR product as the template, and the following additional reagents:

1. 10 µl 10X PCR Buffer (Thermophilic DNA Polymerase 10 x Buffer, magnesium Free M190G, Promega Madison WI, USA)
2. 10 µl 25 mmol MgCl₂
3. 5 µl dNTPs stock solution containing 10 µmol each of dATP, dTTP, dCTP, and dGTP.
4. 1 µl of 100pmol/µl primer HCG-SENTCACC (SEQ ID NO: 18) stock dilution

5. 1 μ l of 100pmol/ μ l primer HCG-ANTISENT (SEQ ID NO: 16) stock dilution
6. 2 μ l TAQ (5 U/ μ l) (Taq DNA Polymerase in Storage Buffer A, Cat No. Mi865, Promega Madison WI, USA)
7. 65 μ l H₂O.

Cycling parameters were as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification with each cycle consisting of 30 sec at 94°C, followed by 1 min at 50°C, and then 2 min at 68°C; and a final extension for 10 min at 72°C.

This PCR product, termed CACCglycalA (SEQ ID NO: 19), was then inserted into the pLenti6/V5 D-TOPO vector according to manufacturer's instructions. This expression construct was named pLenti6/V5-glycalA.

This construct can be used for transient expression of the alpha-FSH subunit in *in vitro* cell culture. Alternatively, where stable expression of the alpha-FSH is desired, the pLenti6/V5-glycalA is transfected into the ViraPower 293FT- Producer cell line, along with the ViralPower packaging mix, to generate a packaged lentivirus (according to manufacturer's instructions: pLenti6/V5 Directional TOPO cloning kit, Invitrogen K4955-10). This packaged retrovirus is used to transduce a mammalian cell line in culture, producing a mammalian cell line that stably expresses the alpha-FSH subunit.

C. Creation of a nucleic acid sequence encoding alpha-FSH + beta-FSH (AB-FSH)

The alpha-FSH and beta-FSH encoding sequences described above were used to create a chimeric nucleic acid molecule alpha-beta FSH, in which the sequences encoding the mature beta-FSH protein (i.e., no signal peptide) were fused to the 3' end of sequences encoding the proprotein of alpha-FSH (i.e., including the signal peptide). The nucleotide sequence of alpha-beta-FSH (AB-FSH; SEQ ID NO: 20) was created using the PCR-based method described above for the production of X1X2 beta human FSH. This nucleotide sequence encodes the AB-FSH polypeptide (SEQ ID NO: 27).

1. Mature beta-FSH

A PCR reaction was performed to generate a nucleic acid sequence encoding mature beta-FSH (i.e., lacking the signal peptide encoding sequences). This PCR reaction was performed using the new forward primer PFMX2 (5'-AAT AGC TGT GAG CTG ACC AA-3'; SEQ ID NO: 21) and the reverse primer PRX2 (SEQ ID NO: 10) on 1 µl of the purified X1X2 PCR product (see Example 1, above) solution as the template. This reaction contained additional reagents and was performed using the cycling parameters as described in Example 1A1 (*Isolation and amplification of two DNA segments each encoding one of two exons of beta-FSH*) above. The resulting PCR product was named S-FSH-B (SEQ ID NO: 22), and confirmed by 2% agarose gel electrophoresis (performed as described above; see Figure 5, lane 2)

2. Proprotein alpha-FSH

A second PCR reaction was performed to generate a nucleic acid sequence encoding the proprotein of alpha-FSH in which the stop codon was removed. Removal of the alpha-FSH stop codon is necessary to prevent premature truncation of the alpha-beta-FSH fusion protein. This PCR reaction was performed using the primer HCG-SENT (SEQ ID NO: 15) and the new primer HCG-ANTISENT/woTAA (5'-AGA TTT GTG ATA ATA ACA AGT ACT GCA GTG G-3'; SEQ ID NO: 23) on the glycalA PCR product (SEQ ID NO: 17) as template. This PCR contained 1 µl glycalA (alpha-FSH) PCR product as the template, and the following additional reagents:

1. 10 µl 10X PCR Buffer (Thermophilic DNA Polymerase 10 x Buffer, magnesium Free M190G, Promega Madison WI, USA)
2. 10 µl 25 mmol MgCl₂
3. 5 µl dNTPs stock solution containing 10 µmol each of dATP, dTTP, dCTP, and dGTP.
4. 1 µl of 100pmol/µl primer HCG-SENT (SEQ ID NO: 15) stock dilution

5. 1 μ l of 100pmol/ μ l primer HCG-ANTISENT/woTAA (SEQ ID NO: 23) stock dilution
6. 2 μ l TAQ (5 U/ μ l) (Taq DNA Polymerase in Storage Buffer A, Cat No. Mi865, Promega Madison WI, USA)
7. 65 μ l H₂O.

Cycling parameters were as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification with each cycle consisting of 30 sec at 94°C, followed by 1 min at 50°C, and then 2 min at 68°C; and a final extension for 10 min at 72°C. The resulting PCR product was named glycalwoTAA (SEQ ID NO: 24; alpha-FSH sequences without the TAA stop codon).

3. alpha-beta-FSH (AB-FSH)

A first PCR was performed to generate an intermediate nucleic acid molecule in which a short segment of 5' mature beta-FSH encoding sequence (S-FSH-B) was linked to the 3' end of the alpha-FSH sequence with the stop codon removed (glycalwoTAA). This PCR was performed using the glycalwoTAA PCR product as template, the forward primer HCG-SENT (SEQ ID NO: 15), and the hybrid reverse primer ABLIGATION (5'-TTG GTC AGC TCA CAG CTA TTA GAT TTG TGA TAA TAA CAA GTA CTG CAG TGG-3'; SEQ ID NO: 25), where the underlined sequence is the reverse complement of primer PFMX2 (SEQ ID NO: 21) and the sequence of primer ANTISENT/woTAA (SEQ ID NO: 23) is in italics. This PCR contained 1 μ l glycalwoTAA PCR product as the template, and the following additional reagents:

1. 10 μ l 10X PCR Buffer (Thermophilic DNA Polymerase 10 x Buffer, magnesium Free M190G, Promega Madison WI, USA)
2. 10 μ l 25 mmol MgCl₂
3. 5 μ l dNTPs stock solution containing 10 μ mol each of dATP, dTTP, dCTP, and dGTP.

4. 1 μ l of 100pmol/ μ l primer HCG-SENT (SEQ ID NO: 15)
stock dilution
5. 1 μ l of 100pmol/ μ l primer ABLIGATION (SEQ ID NO: 25)
stock dilution
6. 2 μ l TAQ (5 U/ μ l) (Taq DNA Polymerase in Storage Buffer
A, Cat No. Mi865, Promega Madison WI, USA)
7. 65 μ l H₂O.

Cycling parameters were as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification with each cycle consisting of 30 sec at 94°C, followed by 1 min at 50°C, and then 2 min at 68°C; and a final extension for 10 min at 72°C. We named the product of this PCR Reaction glycalwoTAAUR (SEQ ID NO: 26).

A second PCR reaction was then performed to generate the final alpha-beta-FSH sequence (AB-FSH) using the glycalwoTAAUR intermediate PCR product (SEQ ID NO: 26) and the S-FSH-B PCR product (SEQ ID NO: 22) as templates. The PCR was performed using the forward primer HCG-SENT (SEQ ID NO: 15) and the reverse primer PRX2 (SEQ ID NO: 10). This PCR reaction contained the following reagents:

1. 10 μ l 10X PCR Buffer (Thermophilic DNA Polymerase 10 x
Buffer, magnesium Free M190G, Promega Madison WI,
USA)
2. 10 μ l 25 mmol MgCl₂
3. 5 μ l dNTPs stock solution containing 10 μ mol each of dATP,
dTTP, dCTP, and dGTP.
4. 1 μ l glycalwoTAAUR intermediate PCR product (SEQ ID
NO: 26)
5. 1 μ l X2 S-FSH-B PCR product (SEQ ID NO: 22)
6. 1 μ l of 100pmol/ μ l primer HCG-SENT (SEQ ID NO: 15)
stock dilution

7. 1 μ l of 100pmol/ μ l primer PRX2 (SEQ ID NO: 10) stock dilution
8. 2 μ l TAQ (5 U/ μ l) (Taq DNA Polymerase in Storage Buffer A, Cat No. Mi865, Promega Madison WI, USA)
9. 65 μ l H₂O

Cycling parameters were as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification with each cycle consisting of 30 sec at 94°C, followed by 1 min at 50°C, and then 2 min at 68°C; and a final extension for 10 min at 72°C. Cycling was performed using a PTC-100TM programmable Thermal Controller (MJ Research Inc, Watertown, Mass, USA).

The product of this PCR was confirmed to be AB-FSH (SEQ ID NO: 20) by 2% agarose gel electrophoresis (performed as described above; see Figure 5, lane 4) and by sequencing of the pLenti/AB-FSH construct (see below) using vector primers.

The AB-FSH PCR product was then cloned into the pLenti6/V5 D-TOPO vector using the pLenti6/V5 Directional TOPO cloning kit according to manufacturer's instructions (Invitrogen K4955-10) as described above (Example 3B. "Alpha-FSH subunit encoding nucleic acid sequence") except that the PCR primers used to add the nucleotide sequence CACC were HCG-SENTCACC (SEQ ID NO: 18) and PRX2 (SEQ ID NO: 10). This expression construct was named pLenti/AB-FSH.

Example 4: Co-expression of alpha-FSH and beta-FSH in mammalian cell culture

The pLenti6/V5-glycalA (pLenti6/V5-D-topo vector containing the glycalA alpha-FSH insert) and the pTPKBFSH (pTarget containing the SDK-X1X2 beta-FSH insert) expression constructs were used for the expression of the complete human FSH hormone in COS-7L (Invitrogen, catalog No. 11622016), and CHO-S (Invitrogen, catalog No. 11619012) cell lines. The alpha-FSH and beta-FSH expression constructs were co-transfected into the various cell lines using the LIPOFECTAMINE 2000 (Invitrogen) transfection reagent according to

manufacturer's instructions. The expression vector pSV- β -Galactosidase (Promega) was included as a transfection control.

Following transfection, the cells were cultured in a selective medium containing 1000 μ g/ml Geneticine and 400 μ g/ml Blasticidin for 7 days. Geneticine-Blasticidine-resistant cells were transferred in a new culture medium containing Geneticine (200 μ g/ml) and Blasticidin (200 μ g/mL), and cultured to a cell density of 10^6 cells/ml. The supernatant of this culture was then tested for the presence of the multisubunit (alpha subunit and beta subunit non-covalently associated) FSH complex.

The presence of the alpha and beta FSH complex was detected using the BIOSOURCE FSH-IRMA Kit (Biosource Europe S.A., Cat # KIP0841-KIP0844), an immunoradiometric assay kit for the quantitation of Follicle Stimulating Hormone (FSH). This FSH-IRMA assay is based on the use of two monoclonal antibodies, one specific for the alpha subunit of FSH and one for the beta subunit of FSH. Thus this assay only detects the complete alpha and beta FSH complex. The supernatant of pLenti6/V5-glycA and the pTPKBFSH co-transfected cells was tested using the FSH-IRMA kit according to manufacturer's instructions. This assay indicated that the supernatant contains alpha and beta FSH complex in an amount of 110 U/ml.

Example 5: Expression of the alpha-beta-FSH fusion protein in mammalian cell culture

The alpha-beta-FSH expression construct pLenti/AB-FSH was transfected into COS-7L cells (Invitrogen, catalog No. 11622016) using the LIPOFECTAMINE 2000 (Invitrogen) transfection reagent according to manufacturer's instructions. The expression vector pSV- β -Galactosidase (Promega) was included as a transfection control.

Following transfection, the cells were cultured in a selective medium containing 400 μ g/ml blasticidin (Gibco) for 7 days. Blasticidin-resistant cells were transferred in a new culture medium containing Geneticine at 200 μ g/ml, and cultured to a cell density of 10^6 cells/ml. These cells were then harvested and lysed. Cells

were lysed by freezing and thawing in 1mL of culture medium. The lysed cells were then centrifuged to pellet the cellular debris. The supernatant was then harvested and tested for the presence of alpha-beta-FSH.

The presence of the alpha-beta-FSH fusion protein was then detected using the BIOSOURCE FSH-IRMA Kit (Biosource Europe S.A., Cat # KIP0841-KIP0844), an immunoradiometric assay kit for the quantitation of Follicle Stimulating Hormone (FSH). This FSH-IRMA assay is based on the use of two monoclonal antibodies, one specific for the alfa subunit of FSH and one for the beta subunit of FSH. The supernatant of lysed pLenti/AB-FSH-transfected COS-7L cells was tested using the FSH-IRMA kit according to manufacturer's instructions. This assay indicated that the the chimeric alpha-beta-FSH protein was present at a concentration of 325 U/ml. Note that for this assay, this results indicates the amount of protein alpha-beta-FSH protein present, but this assay is not truly a functional assay.

To verify that the produced alpha-beta-FSH protein is biologically active, the supernatant of lysed pLenti/AB-FSH-transfected COS-7L cells is tested in the Granulosa assay as described in Example 2, supra. For this assay, 400 µl of GAB assay medium and 60 µL of granulosa cell suspension (~50,000-80,000 viable cells) are added to each well of a 24-well plate. Next, 40 µL of supernatant of lysed pLenti/AB-FSH-transfected COS-7L cells or of various concentrations of positive control recombinant FSH (Organon) are added to each well. The plates are then cultured for 3 days at 37°C in a humidified 5% CO₂ incubator. The supernatant from each well is then harvested, and 10-20 µl of the supernatant assayed for estrogen levels by radioimmunoassay. Estrogen radioimmunoassay is performed using the Spectria RIA kit for estradiol measurement (Orion Diagnostic, Finland), according to the manufacturer's instructions.

For this assay, various concentrations of the positive control recombinant FSH (Organon) are tested and used to calculate a titration curve for FSH activity (recombinant FSH concentration in milliUnits per milliliter, mU/ml, versus estrogen

radioimmunoassay values). This control curve is then used to interpolate the amount of biologically active alpha-beta-FSH. The alpha-beta-FSH of the invention shows a substantially higher activity profile in this assay, as expressed in Units of biological activity per mg of protein, than the currently available recombinant FSH (Organon).

Example 6: Assembly of INF- β / INF- α 2B expression constructs

The sequence of the genomic DNA encoding the INF- β is at Genbank Accession NM 002167. The cDNA sequence of the INF- β is SEQ ID NO: 28 (hereinafter referred to as THIMIOS1) and it corresponds to the above sequence NM 002167 without the stop codon. The sequence of the mature INF- α 2B is a part of Genbank Accession Genbank NM 000605 (hereinafter referred to as PENNY1).

A PCR amplification of beta-FSH encoding sequences

1. Isolation and amplification of two DNA segments each encoding one of two interferons (INF- β and INF- α 2B)

Human genomic DNA was extracted from 50 μ l of total blood using the Nucleo Spin Blood Quick Pure kit (Macherey-Nagel GmbH & Co.). The isolated DNA was then dissolved in 100 μ l of TB Buffer (DNA solution). 10 μ l of this DNA solution was used as template for two independent PCR reactions (5 μ l genomic DNA solution per PCR reaction). The first PCR reaction was performed using primers specific for INF- β .

The forward primer has sequence ATGACCAACAAGTGTCTCCTCCAAATTGCT (hereinafter referred to as THIMIOSF). The reverse primer without stopcodon has sequence GTTTCGGAGGTAACTGTAAAGTCTGTTAAT (hereinafter referred to as THIMIOSR). This PCR reaction amplifies the product CDS of INF- β .

The second PCR reaction was performed using primers specific for the mature chain of INF- α 2B. The forward primer GACGACGACGACAAGTGTGATCTGCCTCAAACCCA (hereinafter referred to as PENNYF) contains 1-5 prime sequencing expressing an enterokinase site; this site

has been added to give the expressed protein molecule the option to be cut, after production, thus providing, in case, two different products.

The mature INF- α 2B sequence containing the enterokinase site (hereinafter referred to as PENNYF) corresponds to SEQ ID NO: 29.

The reverse primer is TCATTCCTTACTTCTTAAAC (hereinafter referred to as PENNYR).

Each independent PCR reaction contained 5 μ l human genomic DNA solution as the template, and the following additional reagents:

1. 10 μ l 10X PCR Buffer (Thermophilic DNA Polymerase 10 x Buffer, magnesium Free M190G; Promega Madison WI, USA)
2. 10 μ l 25 mmol $MgCl_2$
3. 5 μ l dNTPs stock solution containing 10 μ mol each of dATP, dTTP, dCTP, and dGTP.
4. 1 μ l of 100pmol/ μ l forward primer stock dilution
5. 1 μ l of 100pmol/ μ l reverse primer stock dilution
6. 2 μ l TAQ (5 U/ μ l) (Taq DNA Polymerase in Storage Buffer A, Cat No. Mi865, Promega Madison WI, USA)
7. 65 μ l H_2O .

Cycling parameters were as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification with each cycle consisting of 30 sec at 94°C, followed by 30 sec at 50°C, and then 1 min at 72°C; and a final extension for 5 min at 72°C. Cycling was performed using a PTC-100TM programmable Thermal Controller (MJ Research Inc, Watertown, Mass, USA).

Amplification of the INF- β and INF- α 2B products PCR was confirmed by agarose gel (2% in TAE buffer) electrophoresis for 30 min at 125mA using the DNA Molecular Weight Marker ϕ X/Hinc II MK13a (HT Biotechnology Ltd., Cambridge, UK) as control molecular weight markers. The results are shown in Figure 4 (see

Lanes 2 and 3). The products of the PCR reaction were then purified using the PCR Clean-up Kit (Nucleospin Extract, Cat No. 740590.50).

The thus-obtained INF- β / INF- α 2B with enterokinase site corresponds to SEQ ID NO: 30 whereas that without enterokinase site corresponds to SEQ ID NO: 31.

In an alternate strategy the final PCR product was generated by a single three primer PCR using the INF- β and INF- α 2B products as templates and, as PCR primers, THIMIOSF, PENNYR and the ligation primer TINALEME having the sequence reported here-below.

ATCACACTTGTCGTCGTCGTTTCGGAGGTAACCTGTAAGTCT

1. 10 μ l 10X PCR Buffer (Thermophilic DNA Polymerase 10 x Buffer, magnesium Free M190G, Promega Madison WI, USA)
2. 10 μ l 25 mmol MgCl₂
3. 5 μ l dNTPs stock solution containing 10 μ mol each of dATP, dTTP, dCTP, and dGTP.
4. 2 μ l THIMIOS1 PCR product
5. 2 μ l HESEMESA PCR product
6. 1 μ l of 100pmol/ μ l forward primer stock THIMIOSF
7. 1 μ l of 100pmol/ μ l revers primer stock PENNYR
8. 1 μ l of 100pmol/ μ l hybrid primer TINALEME
9. 2 μ l TAQ (5 U/ μ l) (Taq DNA Polymerase in Storage Buffer A, Cat No. Mi865, Promega Madison WI, USA)
10. 65 μ l H₂O

Cycling parameters were as follows: initial denaturation for 2 min at 95°C; 30 cycles of amplification with each cycle consisting of 30 sec at 94°C, followed by 1 min at 50°C, and then 2 min at 68°C; and a final extension for 10 min at 72°C. Cycling was performed using a PTC-100TM programmable Thermal Controller (MJ Research Inc, Watertown, Mass, USA).

Amplification of the product by this PCR reaction was confirmed by agarose (2% in TAE buffer) electrophoresis performed as described above. The product of

this PCR reaction was then purified using the PCR Clean-up Kit (Nucleospin Extract, Cat No. 740590.50) to yield pure DNA in a final volume of 50 μ l. Purity and size of the DNA was confirmed by agarose (2% in TAE buffer) electrophoresis performed as described above. The size of the product was determined by agarose gel electrophoresis.

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

CLAIMS

1. A method for making a polynucleotide (X1X2) comprising two nucleotide segments of interest, X1 and X2, wherein X2 in X1X2 is immediately 3' to X1, from a nucleic acid molecule including X1 and the same or a different nucleic acid molecule including X2, wherein if X1 and X2 originate on the same molecule, they are not contiguous, the method comprising:

(a) amplifying a first double stranded nucleic acid segment X1, which segment comprises a sense and an antisense nucleic acid strand, with a first primer set, which primer set comprises (i) a forward primer, PFX1, which hybridizes to the 3' end of the antisense strand of X1 and (ii) a reverse primer, PRX1, which hybridizes to the 3' end of the sense strand of X1;

(b) amplifying a second double stranded nucleic acid segment, X2, which segment comprises a sense and an antisense nucleic acid strand, with a second primer set, which second primer set comprises (i) a forward primer, PFX2, which hybridizes to the 3' end of the antisense strand of X2 and (ii) a reverse primer, PRX2, which hybridizes to the 3' end of the sense strand of X2;

(c) isolating the X1 and X2 products of steps (a) and (b)

(d) performing PCR in a single reaction vessel, said vessel comprising the isolated X1 and X2 products of step (c) in stoichiometric amounts and primers PFX1, PRX2, and a fusion primer, which fusion primer has the nucleotide sequence of PRX1 preceded at its 5' end by the sequence of the complement of PFX2, said PFX2 complement termed PFX2', wherein PCR performed in this single vessel results in amplification of an intermediate double stranded polynucleotide X1UR, which intermediate comprises a sense and an antisense nucleic acid strand, and which comprises the double stranded nucleic acid segment X1 and a 5' double stranded nucleic acid segment of X2, wherein the 3' end of X1 is fused to the 5' segment of X2, and wherein said reaction also results in amplification of the X1UR intermediate to make X1X2 by denaturing and annealing X1UR and X2 to form annealed species and then extending and amplifying said annealed species using DNA polymerase

possessing both 5'-3' polymerase activity and 5'-3' exonuclease activity and primers PFX1 and PRX2.

2. A method for making a polynucleotide (X1X2) comprising two nucleotide segments of interest, X1 and X2, wherein X2 in X1X2 is immediately 3' to X1, from a nucleic acid molecule including X1 and the same or a different nucleic acid molecule including X2, wherein if X1 and X2 originate on the same molecule, they are not contiguous, the method comprising:

(a) amplifying a first double stranded nucleic acid segment X1, which segment comprises a sense and an antisense nucleic acid strand, with a first primer set, which primer set comprises (i) a forward primer, PFX1, which hybridizes to the 3' end of the antisense strand of X1 and (ii) a reverse primer, PRX1, which hybridizes to the 3' end of the sense strand of X1;

(b) amplifying a second double stranded nucleic acid segment, X2, which segment comprises a sense and an antisense nucleic acid strand, with a second primer set, which second primer set comprises (i) a forward primer, PFX2, which hybridizes to the 3' end of the antisense strand of X2 and (ii) a reverse primer, PRX2, which hybridizes to the 3' end of the sense strand of X2;

(c) isolating the X1 and X2 products of steps (a) and (b)

(d) performing PCR in a single reaction vessel, said vessel comprising the isolated X1 and X2 products of step (c) in stoichiometric amounts and primers PRX1, PFX2, and a fusion primer, which fusion primer has the nucleotide sequence of PFX2 preceded at its 5' end by the sequence of the complement of PRX1, said PRX1 complement termed PRX1', wherein PCR performed in this single vessel results in amplification of an intermediate double stranded polynucleotide DRX2, which intermediate comprises a sense and an antisense nucleic acid strand, and which comprises the double stranded nucleic acid segment X2 and a 3' double stranded nucleic acid segment of X1, wherein the 5' end of X2 is fused to the 3' segment of X1, and said reaction also results in amplification of the DRX2 intermediate to make X1X2 by denaturing and annealing DRX2 and X1 to form annealed species and then

extending and amplifying said annealed species using DNA polymerase possessing both 5'-3' polymerase activity and 5'-3' exonuclease activity and primers PRX1 and PFX2.

3. A method for making a polynucleotide (X1X2) comprising two nucleotide segments of interest, X1 and X2, wherein X2 in X1X2 is immediately 3' to X1, from a nucleic acid molecule including X1 and the same or a different nucleic acid molecule including X2, wherein if X1 and X2 originate on the same molecule, they are not contiguous, the method comprising:

(a) amplifying a first double stranded nucleic acid segment X1, which segment comprises a sense and an antisense nucleic acid strand, with a first primer set, which primer set comprises (i) a forward primer, PFX1, which hybridizes to the 3' end of the antisense strand of X1 and (ii) a reverse primer, PRX1, which hybridizes to the 3' end of the sense strand of X1;

(b) amplifying a second double stranded nucleic acid segment, X2, which segment comprises a sense and an antisense nucleic acid strand, with a second primer set, which second primer set comprises (i) a forward primer, PFX2, which hybridizes to the 3' end of the antisense strand of X2 and (ii) a reverse primer, PRX2, which hybridizes to the 3' end of the sense strand of X2;

(c) amplifying an intermediate double stranded polynucleotide X1UR, which intermediate comprises a sense and an antisense nucleic acid strand, and which comprises the double stranded nucleic acid segment X1 and a 5' double stranded nucleic acid segment of X2, wherein the 3' end of X1 is fused to the 5' segment of X2, with a third primer set, which third primer set comprises (i) PFX1 and (ii) a fusion primer, which fusion primer has the nucleotide sequence of PRX1 preceded at its 5' end by the sequence of the complement of PFX2, said PFX2 complement termed PFX2'; and

(d) making and amplifying X1X2 by denaturing and annealing X1UR and X2 to form annealed species and then extending and amplifying said annealed species using DNA polymerase and primers PFX1 and PRX2.

4. A method for making a polynucleotide (X1X2) comprising two nucleotide segments of interest, X1 and X2, wherein X2 in X1X2 is immediately 3' to X1, from a nucleic acid molecule including X1 and the same or a different nucleic acid molecule including X2, wherein if X1 and X2 originate on the same molecule, they are not contiguous, the method comprising:

(a) amplifying a first double stranded nucleic acid segment X1, which segment comprises a sense and an antisense nucleic acid strand, with a first primer set, which primer set comprises (i) a forward primer, PFX1, which hybridizes to the 3' end of the antisense strand of X1 and (ii) a reverse primer, PRX1, which hybridizes to the 3' end of the sense strand of X1;

(b) amplifying a second double stranded nucleic acid segment, X2, which segment comprises a sense and an antisense nucleic acid strand, with a second primer set, which second primer set comprises (i) a forward primer, PFX2, which hybridizes to the 3' end of the antisense strand of X2 and (ii) a reverse primer, PRX2, which hybridizes to the 3' end of the sense strand of X2;

(c) amplifying an intermediate double stranded polynucleotide DRX2, which intermediate comprises a sense and an antisense nucleic acid strand, and which comprises the double stranded nucleic acid segment X2 and a 3' double stranded nucleic acid segment of X1, wherein the 5' end of X2 is fused to the 3' segment of X1, with a third primer set, which third primer set comprises (i) PRX2 and (ii) a fusion primer, which fusion primer has the nucleotide sequence of PFX2 preceded at its 5' end by the sequence of the complement of PRX1, said PRX1 complement termed PRX1'; and

(d) making and amplifying X1X2 by denaturing and annealing DRX2 and X2 to form annealed species and then extending and amplifying said annealed species using DNA polymerase and primers PRX1 and PFX2.

5. A method for making a polynucleotide (X1X2) comprising two nucleotide segments of interest, X1 and X2, wherein X2 in X1X2 is immediately 3' to X1, from a nucleic acid molecule including X1 and the same or a different nucleic acid

molecule including X2, wherein if X1 and X2 originate on the same molecule, they are not contiguous, the method comprising;

(a) amplifying a first double stranded nucleic acid segment, X2, which segment comprises a sense and an antisense nucleic acid strand, with a second primer set, which second primer set comprises (i) a forward primer, PFX2, which hybridizes to the 3' end of the antisense strand of X2 and (ii) a reverse primer, PRX2, which hybridizes to the 3' end of the sense strand of X2;

(b) amplifying an intermediate double stranded polynucleotide X1UR, which intermediate comprises a sense and an antisense nucleic acid strand, and which comprises the double stranded nucleic acid segment X1 and a 5' double stranded nucleic acid segment of X2, wherein the 3' end of X1 is fused to the 5' segment of X2, with a third primer set, which third primer set comprises (i) a forward primer, PFX1, which hybridizes to the 3' end of the antisense strand of X1 and (ii) a fusion primer, which fusion primer has the nucleotide sequence of a reverse primer, PRX1, which hybridizes to the 3' end of the sense strand of X1, preceded at its 5' end by the sequence of the complement of PFX2, said PFX2 complement termed PFX2'; and

(c) making and amplifying X1X2 by denaturing and annealing X1UR and X2 to form annealed species and then extending and amplifying said annealed species using DNA polymerase and primers PFX1 and PRX2.

6. A method for making a polynucleotide (X1X2) comprising two nucleotide segments of interest, X1 and X2, wherein X2 in X1X2 is immediately 3' to X1, from a nucleic acid molecule including X1 and the same or a different nucleic acid molecule including X2, wherein if X1 and X2 originate on the same molecule, they are not contiguous, the method comprising:

(a) amplifying a first double stranded nucleic acid segment X1, which segment comprises a sense and an antisense nucleic acid strand, with a first primer set, which primer set comprises (i) a forward primer, PFX1, which hybridizes to the 3' end of the antisense strand of X1 and (ii) a reverse primer, PRX1, which hybridizes to the 3' end of the sense strand of X1;

(b) amplifying an intermediate double stranded polynucleotide DRX2, which intermediate comprises a sense and an antisense nucleic acid strand, and which comprises the double stranded nucleic acid segment X2 and a 3' double stranded nucleic acid segment of X1, wherein the 5' end of X2 is fused to the 3' segment of X1, with a third primer set, which third primer set comprises (i) a reverse primer, PRX2, which hybridizes to the 3' end of the sense strand of X2 and (ii) a fusion primer, which fusion primer has the nucleotide sequence of a forward primer, PFX2, which hybridizes to the 3' end of the antisense strand of X2 preceded at its 5' end by the sequence of the complement of PRX1, said PRX1 complement termed PRX1'; and

(c) making and amplifying X1X2 by denaturing and annealing DRX2 and X2 to form annealed species and then extending and amplifying said annealed species using DNA polymerase and primers PRX1 and PFX2.

7. The method of any of claims 1-6 characterized in that the segments X1 and X2 are both included in the same nucleic acid molecule.

8. The method of claim 7 characterized in that said nucleic acid molecule is the genome of a mammalian, preferably a human being.

9. The method of any of claims 1-8 characterized in that said polynucleotide (X1X2) encodes a biologically active polypeptide.

10. The method of claim 9 characterized in that said polynucleotide (X1X2) encodes a polypeptide having the activity of chorionic gonadotropin (CG); luteinizing hormone (LH), follicle stimulating hormone (FSH) or a polypeptide having the activity of both LH and FSH; thyroid stimulating hormone (TSH); alfa-interferon (INF- α), beta-interferon (INF- β) or a polypeptide having the activity of both INF- α and INF- β .

11. The method of any of claims 1-8 characterized in that the segment X1 encodes a signal sequence and X2 encodes a biologically active polypeptide.

12. A method for manufacturing a recombinant protein encoded by the polynucleotide (X1X2) which comprises inserting in a suitable expression vector the polynucleotide (X1X2) obtained by the method according to any of claims 1-6.
13. A nucleic acid molecule comprising the polynucleotide sequence set forth in SEQ ID NO: 20.
14. A vector comprising the nucleic acid molecule of claim 13.
15. A mammalian host cell transformed to express the polynucleotide of SEQ ID NO: 20.
16. A polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 27.
17. An oligonucleotide having the sequence set forth in SEQ ID NO: 11.
18. An oligonucleotide having the sequence set forth in SEQ ID NO: 12.
19. An oligonucleotide having the sequence set forth in SEQ ID NO: 30 or SEQ ID NO: 31.
20. A polypeptide encoded by one of the oligonucleotides according to claim 19.
21. A polypeptide having the activity of the polypeptide according to claim 20.
22. A method for making a polynucleotide (X1X2) comprising two nucleotide segments of interest, X1 and X2, wherein X2 in X1X2 is immediately 3' to X1, from a nucleic acid molecule including X1 and the same or a different nucleic acid molecule including X2, wherein if X1 and X2 originate on the same molecule, they are not contiguous, the method comprising;

(a) amplifying at least one intermediate double stranded polynucleotide selected from the group consisting of X1UR and DRX2, wherein UR is a 5' segment of X2 having at least 12 nucleotides and DR is a 3' segment of X1 having at least 12 nucleotides, in a polymerase chain reaction using the following primer sets:

(i) for X1UR; a forward primer, PFX1, which hybridizes to the 3' end of the antisense strand of X1 and a fusion primer, which fusion primer has the nucleotide sequence of a reverse primer, PRX1, which hybridizes to the 3' end of

the sense strand of X1, preceded at its 5' end by the sequence of the complement of PFX2, said PFX2 complement termed PFX2'; and

(ii) for DRX2, a reverse primer, PRX2, which hybridizes to the 3' end of the sense strand of X2 and a fusion primer, which fusion primer has the nucleotide sequence of a forward primer, PFX2, which hybridizes to the 3' end of the antisense strand of X2 preceded at its 5' end by the sequence of the complement of PRX1, said PRX1 complement termed PRX1';

(b) forming Species A selected from the group of;

(i) the sense strand of X1UR annealed to the antisense strand of X2 where the intermediate is X1UR;

(ii) the sense strand of X1 annealed to the antisense strand of DRX2 where the intermediate is DRX2; and

(iii) the sense strand of X1UR annealed to the antisense strand of DRX2 where the intermediate is X1UR and DRX2;

(c) forming Species B selected from the group of:

(i) the antisense strand of X1UR annealed to the sense strand of X2 where the intermediate is X1UR;

(ii) the antisense strand of X1 annealed to the sense strand of DRX2 where the intermediate is DRX2; and

(iii) the antisense strand of X1UR annealed to the sense strand of DRX2 where the intermediate is X1UR and DRX2; and

(d) by a polymerase chain reaction performed under predetermined polymerization conditions, extending Species A using a polymerase enzyme possessing 5'-3' polymerase activity to form X1X2, and extending Species B using primers PFX1 and PRX2 and a polymerase enzyme possessing both 5'-3' polymerase activity and 5'-3' exonuclease activity to form X1X2.

23. A method for making a polynucleotide (X1X2) comprising two nucleotide segments of interest, X1 and X2, wherein X2 in X1X2 is immediately 3' to X1, from a nucleic acid molecule including X1 and the same or a different nucleic acid

molecule including X2, wherein if X1 and X2 originate on the same molecule, they are not contiguous, the method comprising;

(a) amplifying at least one intermediate double stranded polynucleotide selected from the group consisting of X1UR and DRX2, wherein UR is a 5' segment of X2 having at least 12 nucleotides and DR is a 3' segment of X1 having at least 12 nucleotides, in a polymerase chain reaction using the following primer sets:

(i) for X1UR; a forward primer, PFX1, which hybridizes to the 3' end of the antisense strand of X1 and a fusion primer, which fusion primer has the nucleotide sequence of a reverse primer, PRX1, which hybridizes to the 3' end of the sense strand of X1, preceded at its 5' end by the sequence of the complement of PFX2, said PFX2 complement termed PFX2'; and

(ii) for DRX2, a reverse primer, PRX2, which hybridizes to the 3' end of the sense strand of X2 and a fusion primer, which fusion primer has the nucleotide sequence of a forward primer, PFX2, which hybridizes to the 3' end of the antisense strand of X2 preceded at its 5' end by the sequence of the complement of PRX1, said PRX1 complement termed PRX1';

(b) annealing at least one of the following;

(i) the sense strand of X1UR and the antisense strand of X2 to form Species A;

(ii) the sense strand of X1 and the antisense strand of DRX2 to form Species A; and

(iii) the sense strand of X1UR and the antisense strand of DRX2 to form Species A;

(iv) the antisense strand of X1UR and the sense strand of X2 to form Species B;

(v) the antisense strand of X1 and the sense strand of DRX2 to form Species B; and

(iii) the antisense strand of X1UR and the sense strand of DRX2 to form Species A; and

(c) by a polymerase chain reaction performed under predetermined polymerization conditions, extending Species A using a polymerase enzyme possessing 5'-3' polymerase activity to form X1X2, and extending Species B using primers PFX1 and PRX2 and a polymerase enzyme possessing both 5'-3' polymerase activity and 5'-3' exonuclease activity to form X1X2.

24. The method of claim 22 or 23 characterized in that the segments X1 and X2 are both included in the same nucleic acid molecule.

25. The method of claim 24 characterized in that said nucleic acid molecule is the genome of a mammalian, preferably a human being.

26. The method of any of claims 22-25 characterized in that said polynucleotide (X1X2) encodes a biologically active polypeptide.

27. The method of claim 26 characterized in that said polynucleotide (X1X2) encodes a polypeptide having the activity of chorionic gonadotropin (CG), luteinizing hormone (LH), follicle stimulating hormone (FSH) or thyroid stimulating hormone (TSH).

28. The method of any of claims 22-27 characterized in that the segment X1 encodes a signal sequence and X2 encodes a biologically active polypeptide.

29. A method for manufacturing a recombinant protein encoded by the polynucleotide (X1X2) which comprises inserting in a suitable expression vector the polynucleotide (X1X2) obtained by the method according to any of claims 22-27.

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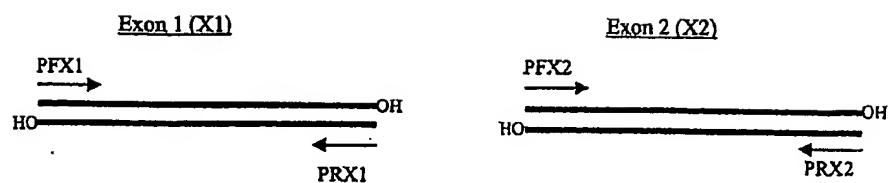


Figure 1

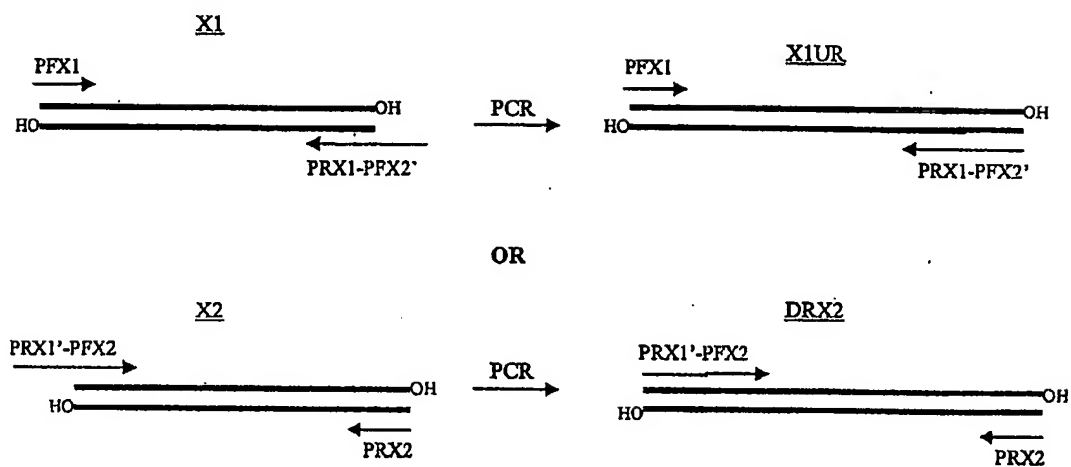


Figure 2

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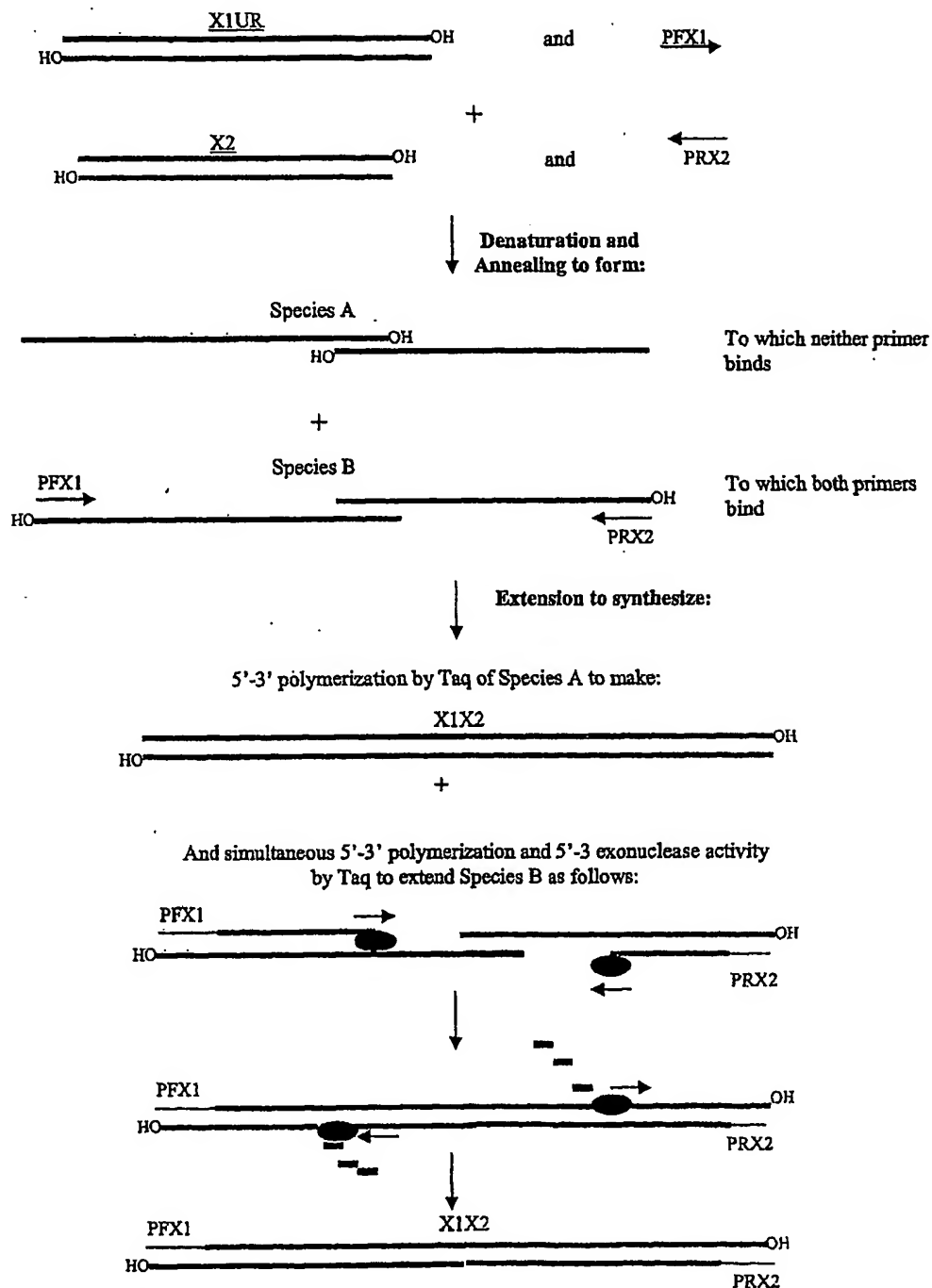


Figure 3

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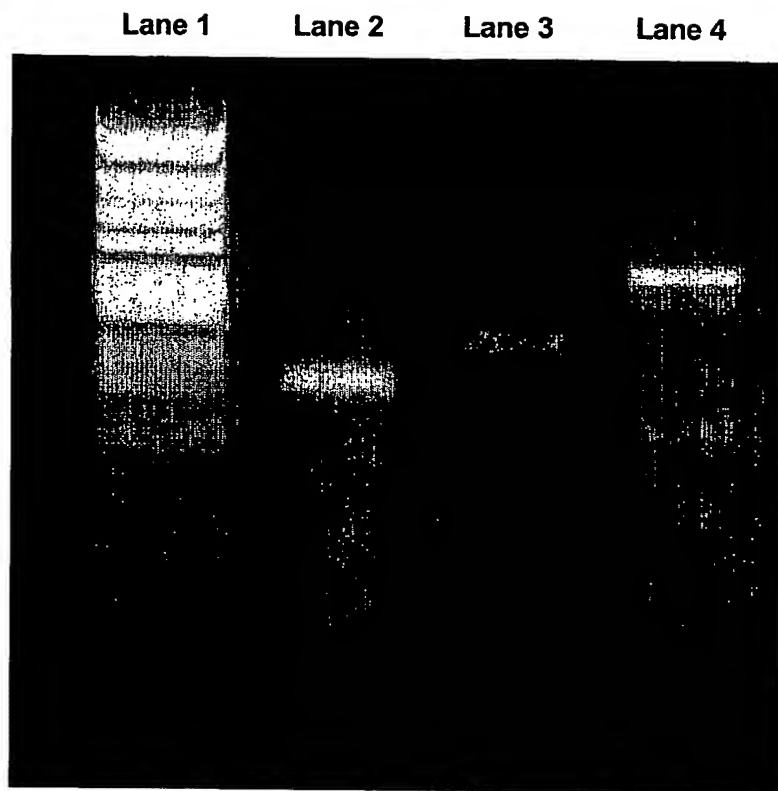


Figure 4

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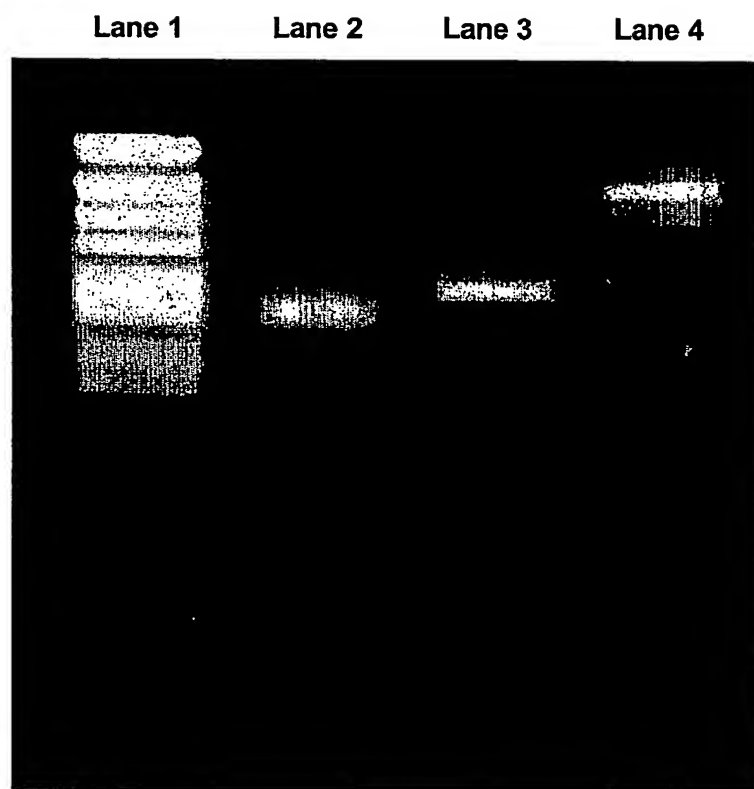


Figure 5

SEQUENCE LISTING

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<120> METHOD OF PRODUCING RECOMBINANT DNA MOLECULES

<130> 04gi30e

<150> US 60/480581

<151> 2003-06-20

<150> US 60/493586

<151> 2003-08-07

<160> 31

<170> PatentIn version 3.1

<210> 1

<211> 1909

<212> DNA

<213> Homo sapiens

<220>

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<222> (1)..(1909)

<223> CDNA sequence for human b-FSH

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attgcaatag agaaagaaga atgtcgtttc tgcataagca tcaacaccac ttggtgtgct	180
ggctactgct acaccagga tctggtgtat aaggagccag ccaggcccaa aatccagaaa	240
acatgtacct tcaaggaact ggtatatgaa acagtgaag tgcccggctg tgctcaccat	300
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gccaggactc catcatgatt cagctctata ttcctaggct tgatttcata aggtttattc	660
agtcttaact cacagacttg tgcttggttt cttctttaaa aatcttagaa atcttctcag	720
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1 5 10 15

tgc tgc aat agc tgt gag ctg acc aac atc acc att gca ata gag aaa 96
Cys Cys Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala Ile Glu Lys
20 25 30

gaa gaa tgt cgt ttc tgc ata agc atc aac acc act tgg tgt gct ggc 144
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35 40 45

tac tgc tac acc agg 159
Tyr Cys Tyr Thr Arg
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 Thr Phe Lys Glu Leu Val Tyr Glu Thr Val Arg Val Pro Gly Cys Ala
 20 25 30
 cac cat gca gat tcc ttg tat aca tac cca gtg gcc acc cag tgt cac 144
 His His Ala Asp Ser Leu Tyr Thr Tyr Pro Val Ala Thr Gln Cys His
 35 40 45
 tgt ggc aag tgt gac agc gac agc act gat tgt act gtg cga ggc ctg 192
 Cys Gly Lys Cys Asp Ser Asp Ser Thr Asp Cys Thr Val Arg Gly Leu
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 Glu Glu Cys Arg Phe Cys Ile Ser Ile Asn Thr Thr Trp Cys Ala Gly
 35 40 45
 Tyr Cys Tyr Thr Arg Asp Leu Val Tyr Lys Asp Pro Ala Arg Pro Lys
 50 55 60
 Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr Val Arg
 65 70 75 80
 Val Pro Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr Pro Val
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atgcagctat ctttctggtc acattgtcgg tgtttctgca tgttctccat tccgctcctg 180

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20 25 30
Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser Gln Pro Gly Ala Pro
35 40 45
Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala Tyr Pro Thr Pro
50 55 60
Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn Val Thr Ser Glu
65 70 75 80
Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val Thr Val Met Gly
85 90 95
Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser Thr Cys Tyr
100 105 110
Tyr His Lys Ser Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala Ile
115 120 125
Glu Lys Glu Glu Cys Arg Phe Cys Ile Ser Ile Asn Thr Thr Trp Cys
130 135 140
Ala Gly Tyr Cys Tyr Thr Arg Asp Leu Val Tyr Lys Asp Pro Ala Arg
145 150 155 160

Pro Lys Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr
 165 170 175
 Val Arg Val Pro Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr
 180 185 190
 Pro Val Ala Thr Gln Cys His Cys Gly Lys Cys Asp Ser Asp Ser Thr
 195 200 205
 Asp Cys Thr Val Arg Gly Leu Gly Pro Ser Tyr Cys Ser Phe Gly Glu
 210 215 220
 Met Lys Glu
 225

<210> 28
 <211> 561
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)..(561)
 <223> cDNA sequence of INF-beta without stop codon

<400> 28
 atgaccaaca agtgtctcct ccaaattgct ctcctgttgt gcttctccac tacagctctt 60
 tccatgagct acaacttgct tggattccta caaagaagca gcaattttca gtgtcagaag 120
 ctcctgtggc aattgaatgg gaggcttgaa tactgcctca aggacaggat gaactttgac 180
 atccctgagg agattaagca gctgcagcag ttccagaagg aggacgccgc attgaccatc 240
 tatgagatgc tccagaacat ctttgctatt ttcagacaag attcatctag cactggctgg 300
 aatgagacta ttgttgagaa cctcctggct aatgtctatc atcagataaa ccatctgaag 360
 acagtcctgg aagaaaaact ggagaaagaa gatttcacca ggggaaaact catgagcagt 420
 ctgcacctga aaagatatta tgggaggatt ctgcattacc tgaaggccaa ggagtacagt 480
 cactgtgcct ggaccatagt cagagtggaa atcctaagga acttttactt cattaacaga 540
 cttacagggt acctccgaaa c 561

<210> 29
 <211> 513
 <212> DNA
 <213> Homo sapiens

<220>
 <221> misc_feature
 <222> (1)..(513)
 <223> INF-alpha-2B sequence with enterokinase site

<400> 29
 gacgacgacg acaagtgtga tctgcctcaa acccacagcc tgggtagcag gaggaccttg 60
 atgctcctgg cacagatgag gagaatctct cttttctcct gcttgaagga cagacatgac 120
 tttggatttc cccaggagga gtttggcaac cagttccaaa aggctgaaac catccctgtc 180
 ctccatgaga tgatccagca gatcttcaat ctcttcagca caaaggactc atctgctgct 240
 tgggatgaga ccctcctaga caaattctac actgaactct accagcagct gaatgacctg 300

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gaagcctgtg tgatacaggg ggtgggggtg acagagactc ccctgatgaa ggaggactcc 360
attctggctg tgaggaaata cttccaaaga atcactctct atctgaaaga gaagaaatac 420
agcccttgtg cctgggaggt tgtcagagca gaaatcatga gatctttttc tttgtcaaca 480
aacttgcaag aaagttaag aagtaaggaa tga 513

```

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<210> 30
<211> 1074
<212> DNA
<213> Homo sapiens

```

```

<220>
<221> misc_feature
<222> (1)..(1074)
<223> INF-beta/INF-alpha-2B sequence with enterokinase site

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<400> 30
atgaccaaca agtgtctcct ccaaattgct ctcctgttgt gcttctccac tacagctctt 60
tccatgagct acaacttgct tggattccta caaagaagca gcaattttca gtgtcagaag 120
ctcctgtggc aattgaatgg gaggcttgaa tactgcctca aggacaggat gaactttgac 180
atccctgagg agattaagca gctgcagcag ttccagaagg aggacgccgc attgaccatc 240
tatgagatgc tccagaacat ctttgctatt ttcagacaag attcatctag cactggctgg 300
aatgagacta ttgttgagaa cctcctggct aatgtctatc atcagataaa ccatctgaag 360
acagtcttgg aagaaaaact ggagaaagaa gatttcacca ggggaaaact catgagcagt 420
ctgcacctga aaagatatta tgggaggatt ctgcattacc tgaaggccaa ggagtacagt 480
cactgtgcct ggaccatagt cagagtggaa atcctaagga acttttactt cattaacaga 540
cttacagggt acctccgaaa cgacgacgac gacaagtgtg atctgcctca aaccacagc 600
ctgggtagca ggaggacctt gatgctcctg gcacagatga ggagaatctc tcttttctcc 660
tgcttgaagg acagacatga ctttgattt ccccaggagg agtttgcaa ccagttccaa 720
aaggctgaaa ccatccctgt cctccatgag atgatccagc agatcttcaa tctcttcagc 780
acaaggact catctgctgc ttgggatgag accctcctag acaaattcta cactgaactc 840
taccagcagc tgaatgacct ggaagcctgt gtgatacagg gggtaggggt gacagagact 900
cccctgatga aggaggactc cattctggct gtgaggaaat acttccaaag aatcactctc 960
tatctgaaag agaagaaata cagcccttgt gcctgggagg ttgtcagagc agaaatcatg 1020
agatcttttt ctttgtcaac aaacttgcaa gaaagttaa gaagtaagga atga 1074

```

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<210> 31
<211> 1059
<212> DNA
<213> Homo sapiens

```

```

<220>
<221> misc_feature
<222> (1)..(1059)
<223> INF-beta/INF-alpha-2B sequence without enterokinase site

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<400> 31
atgaccaaca agtgtctcct ccaaattgct ctctgttgtt gcttctccac tacagctctt 60
tccatgagct acaacttgct tggattccta caaagaagca gcaattttca gtgtcagaag 120
ctcctgtggc aattgaatgg gaggcttgaa tactgcctca aggacaggat gaactttgac 180
atccctgagg agattaagca gctgcagcag ttccagaagg aggacgccgc attgaccatc 240
tatgagatgc tccagaacat ctttgctatt ttcagacaag attcatctag cactggctgg 300
aatgagacta ttgttgagaa cctcctggct aatgtctatc atcagataaa ccatctgaag 360
acagtcctgg aagaaaaact ggagaaagaa gatttcacca ggggaaaact catgagcagt 420
ctgcacctga aaagatatta tgggaggatt ctgcattacc tgaaggccaa ggagtacagt 480
cactgtgcct ggaccatagt cagagtggaa atcctaagga acttttactt cattaacaga 540
cttacagggtt acctccgaaa ctgtgatctg cctcaaacc acagcctggg tagcaggagg 600
accttgatgc tcctggcaca gatgaggaga atctctcttt tctcctgctt gaaggacaga 660
catgactttg gatttcccca ggaggagttt ggcaaccagt tccaaaaggc tgaaaccatc 720
cctgtcctcc atgagatgat ccagcagatc ttcaatctct tcagcacaaa ggactcatct 780
gctgcttggg atgagaccct cctagacaaa ttctacactg aactctacca gcagctgaat 840
gacctggaag cctgtgtgat acagggggtg ggggtgacag agactcccct gatgaaggag 900
gactccattc tggctgtgag gaaatacttc caaagaatca ctctctatct gaaagagaag 960
aaatacagcc cttgtgcctg ggagggtgtc agagcagaaa tcatgagatc tttttctttg 1020
tcaacaaact tgcaagaaag ttaagaagt aaggaaatga 1059